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State University of Iowa	November 18, 1943
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14396

Heightened Thiamine and Choline Requirements in Tropical Heat.

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Keys[1] recently dismissed our findings of heightened thiamine and choline requirements in tropical heat with the brief statement that they were unconvincing. The only basis given for his conclusion was his own study of human subjects exposed to high temperatures *for only one week or less*. In this brief interval of heat exposure, he failed to find any improvement in work performance with accessory vitamin administration (with men on regular army rations).

Reference to the published literature would have shown that no deficiency effects should have been expected in so short a period. Rat growth and food consumption rates begin to show the influence of vitamin deficiency only during the second (or some subsequent) week. This was plainly stated and made clear in the data presented in our paper on heightened thiamine need in the heat.[2] Others[3] have shown that the metabolic adaptation to change in environmental temperature begins in the second week of exposure and is largely accomplished by the end of the third week. Human tests *covering one week or less* therefore have no valid bearing upon the problem and provide no basis for a conviction one way or another.

Since any real heightening of vitamin requirement in tropical heat is a matter of great importance in human and animal nutrition, it is well to eliminate every possible uncertainty. With all vitamins added to our diet mixture and new food given only once or twice a week, it was possible that the destruction of thiamine and choline was more rapid in the feed jars at 90°F than at 68°F and that this may have been the basis of the apparent heightening in requirement. To test this point we kept the diet stocks in a refrigerator and fed only one day's supply at a time, discarding all left-over food. Weanling white rats (Sprague-Dawley males) were divided into groups of 4, 2 to the cage. Daily record was kept of body weight and food consumption. Diets used were the same as previously described[4] except for the addition of vitamin K (2-methyl-1,4-naphthoquinone, 5 mg/kg) and vitamin E (a-tocopherol, 50 mg/kg). The diet containing 1 mg thiamine and 0.75 g choline per kilo of food gives optimal growth at 68°F, while for the same growth rate at 90-91°F 2 mg thiamine and 5 g choline are needed. The following results were obtained after 5 weeks of fresh daily feedings of these diets to 3 series of rats in the heat and cold:

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[1] Keys, Ancel, *Fed. Proc.*, 1943, **2**, 164.

[2] Mills, C. A., *Am. J. Physiol.*, 1941, **133**, 525.

[3] Gelineo, S., *Ann. de Physiol.*, 1934, **10**, 1083; Lee, Robert C., *J. Nutrition*, 1942, **23**, 83.

	Wt gain 3rd, 4th, 5th wks, g	Food eaten in same period, g
Optimal cold room diet:		
Rats kept at 68° F	72.50 ± 4.16	284
Rats kept at 90-91°	42.50 ± 2.12	169
Opt. hot room diet:		
Rats kept at 90-91°F	70.83 ± 1.54	201

The difference between the 2 groups in the hot room (28.33 ± 2.70) would almost never occur by chance alone. This sharply reduced rate of growth, seen when the cold-room diet is used for rats kept in the heat, agrees with all our former results obtained with weekly or semi-weekly feedings. It therefore cannot be vitamin loss on standing exposed to tropical warmth which accounts for the higher thiamine and choline needs in this environment.

Until carefully controlled and adequately planned investigation has shown that thiamine and choline requirements *are not increased* in hot environments, it would seem that our positive findings in the matter should not be dismissed.

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[4] Mills, C. A., *Arch. Biochem.*, 1943, **2**, 159.

14397

The Presence of a Choline-like Substance in Several Injectable Solutions of Liver.*

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It has been shown that experimental polycythemia may be depressed in dogs by the feeding of raw liver or the injection of a purified solution of liver,[1] and also by the feeding of choline hydrochloride.[2] Jacobs[3] has reported that a crude solid liver extract which is effective in the treatment of pernicious anemia contains at least 1% of choline. We have reported the presence of choline in Stomach U.S.P. and the effectiveness of the latter in reducing experimental polycythemia in dogs.[4]

The purpose of this investigation was to determine whether highly purified parenteral solutions of liver contain any choline or choline-like activity.

Methods. Seven injectable solutions of liver, which are available in the market, and which bear labels giving their anti-pernicious anemia potencies in U.S.P. units were obtained. These were tested for possible vasodepressor activity on dogs anesthetized by pentobarbital sodium (30 mg/kg). The extracts were injected intravenously, and the mean carotid arterial pressure was recorded from a mercury manometer.

In further experiments, 6 of the liver extracts were acetylated by the method of Mentzer *et al.*[5] modified to the extent that acetic anhydride was used instead of acetyl chloride. Choline hydrochloride when acetyl-

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* Research paper No. 541, Journal series, University of Arkansas.

[1] Davis, J. E., *Am. J. Physiol.*, 1938, **122**, 397.

[2] Davis, J. E., *Am. J. Physiol.*, 1939, **127**, 322.

[3] Jacobs, H. R., *J. Lab. and Clin. Med.*, 1938, **24**, 128.

[4] Davis, J. E., in press.

[5] Mentzer, C., Corteggiani, E., and Carayon-Gentil, A., *Bull. Soc. Chim. Biol.*, 1939, **21**, 503.

TABLE I.

Changes Produced in the Mean Carotid Arterial Blood Pressure by the Intravenous Injection of Various Liver Extracts. Pressure changes in millimeters of mercury are recorded as rise (+) or fall (—), or no change (0). Dosages of extracts are given in U.S.P. units (u).

				After atropine	
Preparation of liver solution	Units per cc	Untreated liver solution	Acetylated liver solution	Untreated liver extr.	Acetylated liver extr.
I.	20	0 (5u)	— 18 (3u)		0 (3u)
II.	15	0 (5u)	— 60 (7.5u)		0 (7.5u)
			— 49 (5u)		
			— 23 (3u)		0 (3u)
			— 13 (1u)		
			— 12 (2u)		
III.	10	0 (2.5u)	— 30 (2.5u)		0 (2.5u)
			— 76 (4u)		+9 (4u)
			— 51 (1.6u)		
IV.	5	0 (5u)	— 70 (8u)		0 (8u)
			— 26 (7u)		0 (7u)
V.	4	—80 (6u)	— 90 (1u)	—72 (6u)	—15; +20 (1u)
			— 90 (2u)		—26; +44 (2u)
VI.	2	—48 (0.4u)	—110 (0.8u)	—63 (2u)	—38 (1u)
VII.	1	—44 (1u)	not tested	—27 (1u)	not tested

ated by this method showed the same vasodepressor activity as pure acetylcholine in similar concentration. The acetylated extracts were tested for vasodepressor activity both before and after atropinization of the animals.

Results. Table I shows that the 4 most potent preparations (*i.e.*, containing 5 or more U.S.P. units per cc according to their labels) produced no significant change in blood pressure when injected intravenously into dogs in doses of 2.5 to 5 units. After acetylation, however, these same extracts caused significant reductions of blood pressure. No fall of blood pressure occurred when these acetylated extracts were given after the intravenous injection of atropine sulfate (0.5 mg per kg).

The other 3 preparations, with labeled antianemic potencies of less than 5 units per cc, caused marked decreases of blood pressure following the intravenous injection of even small doses (Table I). The vasodepressor activity of 2 of these extracts was tested after acetylation, and appeared to be increased thereby. Atropinization of the animals inhibited or greatly decreased the vasodepressor activity of these 2 extracts. In one experiment the injection of 2 mg of acetylcholine after atropine, caused an exact duplication of the blood pressure record resulting from the injection of 1 unit of acetylated solution number 5 (brief fall of 15 mm followed by a rise to 20 mm above normal).

Extracts Nos. 5, 6 and 7 (untreated) caused significant decreases in blood pressure when injected after atropinization of the animals.

Fig. 1 shows blood pressure tracings which illustrate the kind of records obtained in these experiments.

Discussion. No real correlation was found to exist between antianemic unitage and blood pressure lowering activity in these extracts except perhaps in one experiment where acetylated extracts Nos. 1, 2, and 3 given in doses of 2 or 3 units produced somewhat comparable falls in pressure.

The fact that acetylation produced a blood pressure lowering action in extracts 1-4, and increased that activity already present in the others, and that the action is abolished or diminished by atropine—leads us to believe that choline or a choline-like substance is present in all of the liver extracts tested.

That the acetylation of substances other than choline can result in a product with

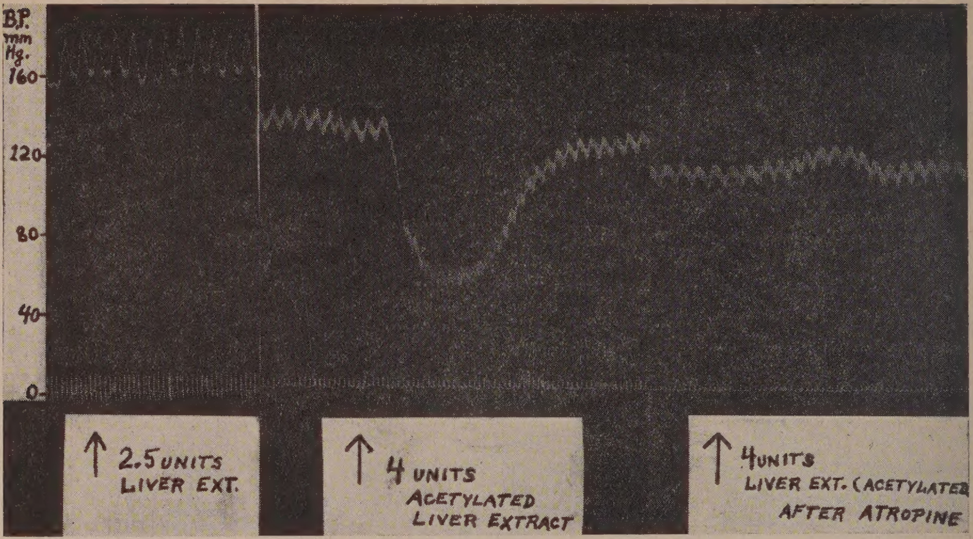

Fig. 1.
Typical Blood Pressure Record Following the Intravenous Injection of Untreated and Acetylated Liver Extracts.

Tracing at left shows the effect of 0.25 cc of a brand of purified solution of liver upon the blood pressure of a dog. The same brand of liver extract (Number III; Table I) was acetylated and injected into another dog both before and after atropinization. Time is recorded in seconds.

increased vasodepressor action has been shown by Hunt[6] and by Dale.[7] However, few such substances are known to be present in the animal body, most of them being products of laboratory synthesis.

Conclusion. Six injectable liver extracts were found to contain choline or a choline-like substance, since their acetylation produced vasodepressor activity which was antagonized by atropine.

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[6] Hunt, R., *J. Pharm. and Exp. Therap.*, 1914, **6**, 477.

[7] Dale, H. H., *J. Pharm. and Exp. Therap.*, 1914, **6**, 147.

14398 P

Phosphatase Activity of Human Erythrocytes.

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Evidence has accumulated which indicates that phosphatases occur in the erythrocytes of dog, beef, horse, rabbit, rat, and man. To provide further information about phosphomonoesterase occurring in the normal human red blood cells the enzymatic activity of red cell hemolysates was studied as to (1) the presence of alkaline and acid phosphatase, (2) the effect of fluoride and magnesium upon the enzymatic activity, (3) the relation of erythrocyte phosphatase to plasma phosphatase.

Procedure. The erythrocytes were washed 3 times with normal saline. The washed cells obtained from x cc of oxalated or heparinized blood were hemolyzed by adding distilled water to bring the total volume of the hemolysate up to 1.5 times x cc. If the hematocrit value is known the phosphatase activity of the erythrocytes can be calculated from the activity of the hemolysates. The phosphatase activity of the hemolysate as well as of the plasma was tested by determining the amount of phosphorus (according to Fiske and Subbarow[1]) liberated from a sodium glycerophosphate substrate, or by measuring the amount of phenol freed from a disodium phenylphosphate substrate, according to King and Armstrong.[2] Michaelis'[3] veronal-acetate buffer was used preferably, since certain buffer salts interfere with the colorimetric determination of phenol. In a few of the experiments with glycerophosphate as substrate, citrate and veronal buffers were used. The results are expressed in mg of phosphate or phenol liberated per 100 cc of hemolysate or plasma during one hour of incubation at 37°C.

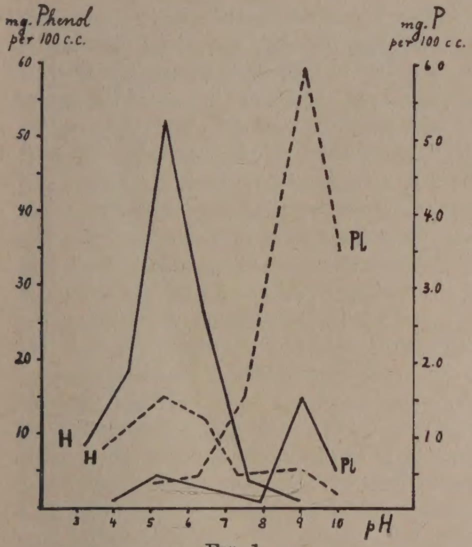

FIG. 1.

Phosphatase activity per 100 cc of human red cell hemolysate (H) and of plasma (Pl) with relation to $[H^+]$-concentration. Incubation at 37°C for one hour.

Broken lines: Phosphorus, liberated from buffered β-glycerophosphate. Buffer: veronal or citrate.

Solid lines: Phenol, liberated from buffered phenylphosphate. Buffer: veronal-acetate.

Results. (1) Phosphatase activity of red cell hemolysates was found to be constantly present (Fig. 1). Strong activity was observed between pH 4.8 and pH 6.1. Below and above this zone of acidity hydrolysis drops abruptly. The optimum pH of the phosphatase of human erythrocytes was found around pH 5.3. Its action upon β-glycerophosphate is slow compared to the strong action upon disodium monophenylphosphate. A second zone of optimal activity at alkaline reaction as reported by Roche and Bullinger[4] for horse blood cells, was missing. If confirmed, this result would preclude the occurrence of an "alkaline phosphatase" in human erythrocytes.

(2) The inhibiting effect of sodium fluoride upon the activity of the intracellular phosphatase was found to be only very slight. If the concentration of fluoride during incubation was M/100 to M/200, the greatest decrease of activity, observed at pH 5.3, was not more than 10.2%. The average decrease was 6.2%. The strong inhibition by fluoride of the plasma phosphatase at pH 5.0 (Gutman[5]) could be confirmed constantly. Experiments failed to show a definite effect of magnesium chloride upon the phosphomonoesterase activity of red cell hemolysates. The action of magnesium chloride was tested—

(a) In final concentrations ranging from M/100 to M/800, at the optimal pH of 5.3. Stronger Mg-solutions caused a precipitation in the substrate-buffer mixture.

(b) With magnesium chloride concentrations kept between M/250 and M/300, but the acidity varying from pH 3.4 to pH 8.9. If changes took place they were slight increases of hydrolysis at the optimal pH of 5.3. In no instance was an increase of enzymatic activity observed as recorded by Jenner and Kay[6] for red cells of rat, dog, beef, and rabbit.

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[1] Fiske, C. H., and Subbarow, V., *J. Biol. Chem.*, 1925, **66**, 375.

[2] King, E. J., and Armstrong, A. R., *Can. Med. Assn. J.*, 1934, **31**, 376.

[3] Michaelis, L., *Bioch. Z.*, 1931, **234**, 139.

[4] Roche, J., and Bullinger, E., *Enzymologica*, 1939, **7**, 278.

[5] Gutman, A. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 513.

[6] Jenner, H. D., and Kay, H. D., *J. Biol. Chem.*, 1931, **93**, 733.

(3) By drawing the curves of activity obtained by intracellular and extracellular blood phosphatases it is possible to demonstrate the relation of these enzymes to each other (Fig. 1). At alkaline reaction the plasma phosphatase activity is far superior to the erythrocyte phosphatase, whereas on the acid side of neutrality the red cell phosphatase activity is predominant. When acting on β-glycerophosphate the "alkaline" plasma phosphatase, at the optimal pH, will liberate four times as much phosphate as the "acid" erythrocyte phosphatase at its optimal pH. But when allowed to act upon phenylphosphate the erythrocyte phosphatase will free about four times as much phenol as plasma phosphatase, both acting as their respective optimal pH

14399 P

Effect of X-Rays on Erythrocytes.

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Earlier attempts to elicit hemolysis *in vitro* by means of X-ray radiation have been almost uniformly negative. Rare positive results relate to delayed hemolysis beginning 15-20 hours (Ting and Zirckle[1]) or more (Holthusen[2]) after irradiation. Levin and Piffault[3] alone observed immediate hemolysis with doses of 1.5 × 10^6 r. The experiments reported here were undertaken in order to ascertain the X-ray doses which induce immediate hemolysis under different conditions.

Fresh rabbit blood was defibrinated, washed twice in saline and irradiated at different degrees of dilution in saline and other vehicles. Small hanging drops under mica slips were irradiated and the effects observed with the microscope.

Irradiation was carried out by means of a demountable X-ray tube with copper anode at a tension of 35 K.V. and a current of 40 M.A. The window was aluminium foil 30 μ in thickness; the mica slip for the hanging drop was 0.02-0.025 mm thick. The X-ray intensity at the distance of the hanging drop—3.5 mm—was about 95,000 r/min.

In a series of tests to determine the doses inducing immediate hemolysis, *i.e.*, hemolysis completed within the time of the irradiation, erythrocytes in saline or physiological glucose solution were irradiated in concentrations of 0.1%, 0.5%, 1%, and 5%. At 1% erythrocyte concentration, serum was also added to the saline in some experiments. The results obtained were satisfactorily constant. The averages are set down in Table I.

The experiments show that the doses necessary in order to induce immediate hemolysis are very high. A noteworthy feature is the importance of the erythrocyte concentration in glucose. Within the erythrocyte concentration range of 0.1% to 5%, the effect of radiation in this medium is augmented by decrease in concentration. At 5% erythrocyte concentration in physiological glucose solution the dose producing immediate hemolysis is 6 times that necessary for an erythrocyte concentration of 0.1%. The dose which produces hemolysis at 5% erythrocyte concentration in glucose, furthermore, is twice that producing this effect at the same erythrocyte concentration in saline.

An unexpected and hitherto unknown effect was observed on erythrocytes irradiated in saline at erythrocyte concentrations less than 0.5%. As may be seen in Table I, the immediate hemolysis dose for 1% and 5% of erythrocytes in saline is 3.5 × 10^6 r. At 0.5% concentration the same effect is pro-

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[1] Ting, T. P., and Zirckle, R. E., *J. Cell. and Comp. Physiol.*, 1940, **16**, 189.

[2] Holthusen, H., *Strahlenther.*, 1922, **14**, 561.

[3] Levin, B., and Piffault, C., *C. R. soc. biol., Paris*, 1934, **116**, 1324.

TABLE I.
Average Doses in Million r Producing Complete Hemolysis of Rabbit Erythrocytes.

	Dilutions			
Medium	0.1%	0.5%	1%	5%
Saline	No hemolysis to 7 × 10^6 r	2.0	3.5	3.5
Glucose	1.25	2.0	3.5	7.0
Saline plus 25% serum	1.5			

duced at 2 × 10^6 r. It could be expected, therefore, that at 0.1% erythrocyte concentration an even smaller dose would cause hemolysis. In fact, hemolysis could not be induced at this cell concentration even by 7 × 10^6 r. Nor could hemolysis be induced, furthermore, by repetition of the irradiation after an interval of 24 hours. The impression was obtained that erythrocytes in suspensions of 0.1% in saline, irradiated with 2 × 10^6 r, became "stable" and could tolerate 7 × 10^6 r and more.

The phenomenon of hemolysis-stability (H-St.) does not occur under the conditions mentioned above, if glucose or saline plus serum is used as medium. In saline it does not occur if an erythrocyte concentration of 0.5% or more is employed. The noted influence in our experiments with erythrocytes of the concentration of the irradiated object and of the nature of the medium find an analogy in results obtained with other test objects. W. M. Dale[4,5] demonstrated the influence of the concentration of the irradiated object ("dilution effect") and the inhibitory influence of certain medium constituents ("protective effect") for the case of enzymes and other substances. Similar observations have been recorded with enzymes by Goldhaber and Leibowitz[6] and with virus by Lacassagne and Nyka[7], Friedewald and Anderson[8] and Luria and Exner[9]. Recently, Evans, Slaughter, Little and Failla[10] showed in experiments on Arbacia sperm and eggs that irradiation of the sperm can prevent fertilization and that the dose effective in this respect depends on the concentration of the sperm as well as on the medium. To explain such phenomena Dale and other investigators assume that the primary effect of radiation is an action on the milieu and that the reaction of the irradiated object is a secondary result. A similar relation appears to hold for irradiated erythrocytes.

Summary. The doses of X-rays which produce immediate hemolysis *in vitro* have been determined for different conditions. The X-ray dose producing complete hemolysis is dependent on the concentration of the irradiated erythrocyte suspension and on the medium employed. Erythrocytes suitably irradiated at high dilutions (0.1%) in saline become stable to X-ray hemolysis.

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[4] Dale, W. M., *Biochem. J.*, 1940, **34**, 1367.

[5] Dale, W. M., *Brit. J. Radiol.*, 1943, **16**, 171.

[6] Goldhaber, G., and Leibowitz, S., *Nature*, 1943, in press.

[7] Lacassagne, A., et Nyka, W., *C. R. soc. biol., Paris*, 1938, **128**, 1938.

[8] Friedewald, D. F., and Anderson, R. S., *J. Exp. Med.*, 1941, **74**, 463.

[9] Luria, S. E., and Exner, F. M., *Proc. Nat. Acad. Sc.*, 1941, **27**, 370.

[10] Evans, T. C., Slaughter, J. C., Little, E. P., and Failla, G., *Radiology*, 1942, **39**, 663.

14400 P

Observations on Acquired Cellular Resistance to Equine Encephalomyelitis Virus.

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In animals vaccinated with formalin-inactivated Eastern (E.E.E.) or Western (W.E.E.) viruses, degree of resistance to intracerebral inoculation of homologous virus is correlated with titer of neutralizing antibody in the serum[1] and with neutralizing capacity of cerebrospinal fluid.[2] In contrast to this specific immunity, a non-specific type of resistance has been observed which cannot be explained on the basis of serological findings. It has been encountered only in animals whose CNS has been previously in contact with certain encephalitic viruses. For study of this phenomenon animals were prepared in the following manner.

"Abortive Infections" with Equine Encephalomyelitis Virus in Guinea Pigs and Rabbits. Guinea pigs vaccinated with formalin-inactivated W.E.E. virus were, after suitable intervals, injected intracerebrally with 1 to 1,000 m.l.d. of W.E.E. virus. In vaccinated animals which survived, the response during the first 24 hours after inoculation was indistinguishable from that of non-vaccinated control animals: a steep rise in temperature occurred, first observed 2 hours after inoculation, which persisted for about 24 hours. While in control animals fever lasted until prostration or death, in immune animals the temperature dropped after 24 to 30 hours and remained normal thereafter. In both normal and vaccinated guinea pigs the intensity of febrile reaction on the first day was proportional to the amount of virus injected. Rabbits vaccinated against E.E.E. or W.E.E. reacted similarly when tested with homologous virus. No such characteristic rise in temperature occurred in guinea pigs or rabbits after intracerebral inoculation of formalin-inactivated W.E.E. virus, of 1% normal mouse brain suspension, or of a neutral mixture of W.E.E. virus and homologous antiserum.

It was concluded that the "fever spike" of vaccinated animals reflected viral action on susceptible tissue and may indicate an "abortive infection."

Resistance of Guinea Pigs and Rabbits after Abortive Infection with E.E. Virus. Guinea pigs vaccinated with formalin-inactivated W.E.E. virus remained fully susceptible to intracerebral inoculation of E.E.E. virus. Others, similarly vaccinated but in addition convalescent from an abortive infection with W.E.E. virus, resisted 2 weeks later intracerebral re-inoculation of 10 or 1,000 m.l.d. of E.E.E. virus. The degree of this non-specific resistance depended on the intensity of the abortive infection. Similar observations were made in rabbits.

Abortive infection with W.E.E. virus rendered guinea pigs resistant to New Jersey strain of vesicular stomatitis virus injected intracerebrally. This resistance was less pronounced than that to E.E.E. virus, and when the interval between the two inoculations was 2 weeks, death occurred, as a rule, although after considerable delay. At 3 weeks, full susceptibility was restored.

Resistance in Mice. (1) Mice, after vaccination with formolized W.E.E. virus and intracerebral test inoculation with homologous virus, were found to resist 10,000 to 100,000 cerebral m.l.d. of E.E.E. virus, while others which had been vaccinated but not tested remained fully susceptible to E.E.E. virus. (2) Mice inoculated intracerebrally with 10 m.l.d. of Theiler's virus (T.V.) developed during the incubation period (13-14 days) an increasing degree of resistance to W.E.E. virus, as shown in Table I. Several mice paralyzed by T.V. for 8 to 9 days were re-inoculated with 10 to 100 m.l.d. of W.E.E. virus and survived.

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[1] Morgan, I. M., and Olitsky, P. K., *J. Immunol.*, 1941, **42**, 445.

[2] Morgan, I. M., Schlesinger, R. W., and Olitsky, P. K., *J. Exp. Med.*, 1942, **76**, 357.

TABLE I.
Fate of Mice Inoculated Intracerebrally with Theiler's Virus and Re-inoculated at Varying Intervals with W.E.E. Virus.

Outcome	Dilution W.E.E. inoculated	Interval between T.V. and W.E.E. virus in days					Total 4-10-day intervals	Total controls‡
		2	4	6	8	10		
Dead of W.E.E. infection	10^{-3}	N.T.	N.T.	N.T.	N.T.	1/4	1/4	N.T.
	10^{-5}	4/5*	5/5	3/5	1/5	0/5	9/20	19/20
	10^{-7}	5/5	0/5	0/5	0/4	N.T.	0/14	17/20
Theiler's disease	10^{-3} 10^{-5} 10^{-7}	1†	5	7	8	8	28/28	20/20

* 4/5 = 4 of 5 inoculated mice died of W.E.E. infection.

† 1 = All mice inoculated with both viruses which did not die of W.E.E. became paralyzed with Theiler's disease after the usual incubation period.

‡ = Control mice receiving at same time as test mice W.E.E. virus or T.V.

N.T. = Not tested.

Discussion. The experimental results recall the interference phenomena described for other neurotropic viruses.[3,4,5] Although "cross-immunity" between Eastern and Western equine encephalomyelitis had been observed before in horses[6,7] and in guinea pigs and rabbits,[8] the conditions under which it arose had not been defined. Non-specific resistance, as described, developed following exposure of the CNS to virus and was demonstrated in the absence of neutralizing antibody; it is believed, therefore, to be due to a change in the susceptibility of the cell. This differentiates it from specific immunity induced by vaccination[1,2] and by passive immunization.[8,9] Attempts are under way to demonstrate resistance to equine encephalomyelitis virus following intracerebral inoculation of inactivated virus. It has been shown for bacteriophage[10] and for influenza virus[11,12] that ultraviolet irradiation does not destroy the interfering capacity. A fuller description will be published elsewhere; meanwhile, the practical significance of the findings should be pointed out. An animal, vaccinated against one of the equine viruses is specifically immune, but the specificity of its immunity is masked, at least temporarily, once it has been tested with an intracerebral challenge dose. This should be borne in mind when vaccinated and tested animals are used for the identification of newly isolated strains of virus.

Summary. Acquired, non-specific cellular resistance to equine encephalomyelitis virus has been described and differentiated from specific immunity induced by vaccination or passive immunization.

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[3] Dalldorf, G., Douglass, M., and Robinson, H. E., *J. Exp. Med.*, 1938, **67**, 333; Dalldorf, G., and Douglass, M., Proc. Soc. Exp. Biol. and Med., 1938, **39**, 294; Dalldorf, G., *J. Immunol.*, 1939, **37**, 245.

[4] Theiler, M., *Medicine*, 1941, **20**, 443.

[5] Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1942, **76**, 127.

[6] Records, E., and Vawter, L. R., *J. Am. Vet. Med. Assn.*, 1934, **85**, 89.

[7] Meyer, K. F., Wood, F., Haring, C. M., and Howitt, B., Proc. Soc. Exp. Biol. and Med., 1934, **32**, 56.

[8] Howitt, B. F., *J. Immunol.*, 1935, **29**, 319.

[9] Olitsky, P. K., Schlesinger, R. W., and Morgan, I. M., *J. Exp. Med.*, 1943, **77**, 359.

[10] Luria, S. E., and Delbrück, M., *Arch. Biochem.*, 1942, **1**, 207.

[11] Henle, W., and Henle, G., *Science*, 1943, **98**, 87.

[12] Ziegler, J. E., Jr., personal communication.

14401

Nerve Reunion with Sleeves of Frozen-Dried Artery in Rabbits, Cats and Monkeys.*

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Sutureless nerve reunion by arterial cuffs in the rat was described in an earlier paper.[1] Instead of live arteries, frozen-dried ones may be used after rehydration. A more recent analysis[2] revealed the reasons for the superiority of this method. Evidence has now accumulated to show that it is equally satisfactory in larger mammals. This conclusion is based on a total of 132 arterial nerve splices in rabbits (21), cats (30) and monkeys (81).

Material and Methods. The nerves used were mostly the peroneal, tibial, or sciatic. No distinction will be made here between simple reunion of severed stumps and reunion with the intercalation of a nerve graft, mostly of frozen-dried nerve (see following article). For arteries, the aorta or carotid was used in most cases; the former is preferable for its lack of musculature. Freezing, drying, storing, and rehydration were done as previously reported,[3] always with strict aseptic precautions. Elasticity, consistency, and histologic character are not noticeably affected by this treatment. For the reunion of severed nerve stumps, arterial segments of matching caliber and measuring from 6 to 10 diameters in length were fitted over the nerve ends by the following improved technic (Fig. 1):

First, the sleeve is pulled over one nerve end with the instrument illustrated in Fig. 1A-C. A stainless steel tube (a) contains a movable flexible wire (b), one end of which rests against a coil spring (c), while the other end is fastened to a chuck (d) ending in two half-cylinders (e_1 and e_2) of the same diameter as tube a, which act as a clutch. They are manipulated through lever f: depressing the lever (Fig. 1A) produces retraction of the conical chuck and closure of its jaws; on releasing the lever, spring c forces the end piece out and the clutch (e) opens owing to the elastic spread of the jaws (d). When the lever is depressed and the open end plugged by a fitting cone (g), the instrument forms a continuous pointed rod (Fig. 1A). In this condition, it is threaded through the lumen of the arterial segment, and the latter is slipped over the shaft a. The lever is then released, cone g is removed, and the free nerve end is grasped between the jaws of the clutch by depressing the lever again. The sleeve can now easily be slipped back from the shaft onto the nerve. This corresponds to stages a-e of our earlier practice (Weiss,[1] Fig. 1). In a second step, the other nerve stump is introduced into the open end of the sleeve. To facilitate this, the latter is widened and held open by a "spreader" (Fig. 1D, E), consisting of 2 curved metal strips (h) attached to the ends of a steel spring bow (i). Instruments of several sizes were available to fit different nerves.†

It is advantageous to leave a small blood-filled gap between the nerve ends in the sleeve. When grafts are used, their length can be adjusted so as to avoid either slack or exces-

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* This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago; also aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. The monkey experiments were carried out at the Yerkes Laboratories of Primate Biology, Orange Park, Florida, and I am greatly indebted to Dr. K. S. Lashley for his generous contribution of laboratory facilities. Technical assistance by Dr. R. W. Sperry and Dr. A. C. Taylor is also gratefully acknowledged.

[1] Weiss, P., *Arch. Surgery*, 1943, **46**, 525.

[2] Weiss, P., and Taylor, A. C., *Arch. Surgery*, 1943, **47**, 419.

[3] Weiss, P., and Taylor, A. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 326.

† The instruments were designed jointly with Dr. A. C. Taylor who also did the actual constructing.

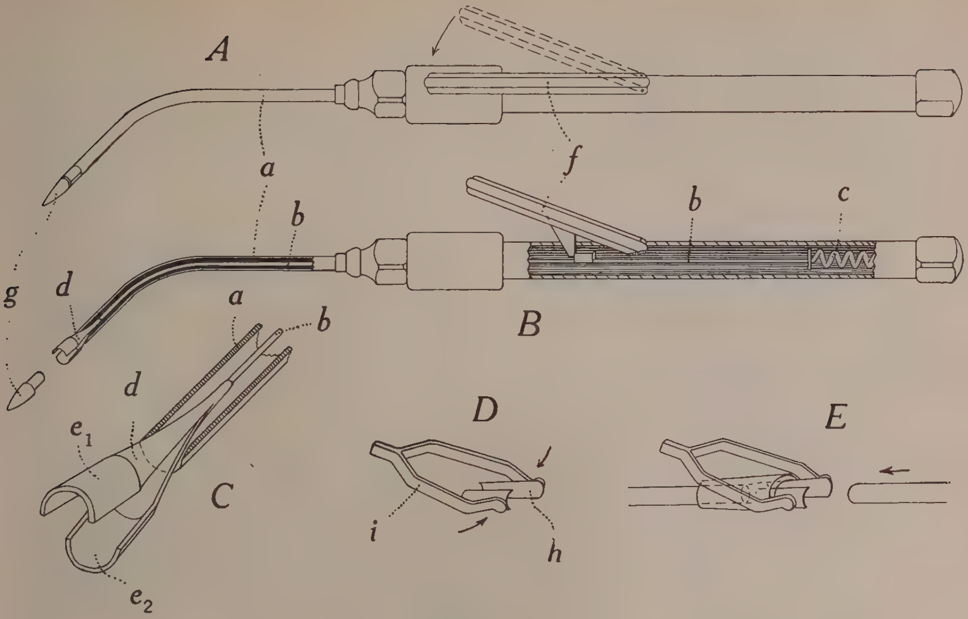

FIG. 1.
Splicing instrument. Explanation in text.

sive stretch, and no further attachment between sleeve and nerve is necessary. The same applies to nerve ends approximated under moderate stretch. Higher degrees of stretch require special provisions (see below). No sutures have been used in any of the cases dealt with in this report: the elastic sleeve, sealed to the nerve by clotting blood, was the only link. No casts or other means of restraining active and passive use of the operated limbs were employed. This placed a severe test upon the arterial unions.

The animals (rabbits, cats, spider monkeys, and rhesus monkeys) have been under observation for up to 10 months. Histological studies thus far completed include 4 rabbit, 8 cat, and 25 monkey splices, in addition to 30 monkey biopsies.

Results. Only in one rabbit and one monkey nerve has the arterial link failed to hold. In all other cases, nerve regeneration has occurred much in the same way as described for the rat under similar conditions. Criteria of success were the rate and completeness of functional recovery and the histological appearance of the union. From the functional standpoint, the results were optimal; histologically, there was a greater proportion of cases with some fiber entanglement than was seen in the rat—a difference readily accounted for by the greater variability in the operative handling of larger nerves. An average case is illustrated in Fig. 2 showing a sleeve-spliced rabbit sciatic nerve 2½ months after the operation; it represents the average in that the line of junction is more evident than in optimal cases, yet less than in the worst cases.

Nerves properly operated upon regenerate in the orderly fashion peculiar to arterial sleeve splicing:[1,2] the great mass of the regenerating fibers pass straight, unbranched, unobstructed across the gap into the distal stump, and there is neither fibrosis nor neuroma formation. This orderly regeneration pattern makes it possible for the majority of fibers of a given fascicle to remain together and, therefore, to reinnervate a relatively localized muscle group, instead of becoming dispersed over the whole denervated periphery at random, as commonly happens after ordinary sutures. As an experimental test of this fact, the following example may be cited:

The right tibial nerve of a spider monkey was severed in the proximal half of the thigh,

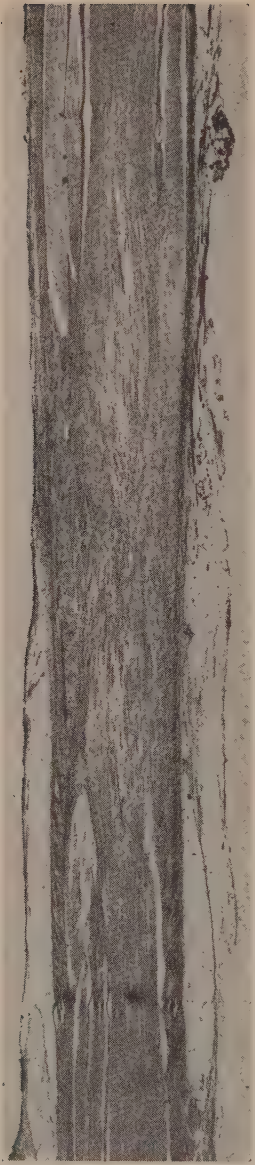

Fig. 2.

Rabbit sciatic nerve 2½ months after reunion with sleeve of frozen-dried artery. Bodian impregnation. × 12. This illustrates an average case considerably below optimum.

and the ends were spliced with a segment of frozen-dried artery from a spider monkey, which had been stored for 2 months, then rehydrated and kept in Ringer's solution in the refrigerator for 2 days before use. (The right peroneal nerve, likewise severed, had received a graft). When examined 6 months after the operation, motor function was completely recovered. The site of the nerve was then exposed and the tibial trunk proximal to the splice was split into 8 constituent fascicles. By stimulating these individually with induction shocks, 6 different and distinct motor effects were obtained, which demonstrates the preservation of fascicular topography in regeneration through the splice. The combined calf muscles of this animal weighed 56 g on the regenerated side and 52 g on the control side.

Branching and commingling of fibers at the junction result from defective operations, *e.g.*, sagging arteries; slack nerves; overhanging epineurium or perineurium. Aside from greater irregularity of the fiber courses, regeneration seemed, however, unimpaired even in these instances.

The frozen-dried arterial sleeves persist as such for many months, perhaps indefinitely. They become partially repopulated by host cells. In contrast to live sleeves, their walls absorb a certain number of regenerating fiber branches. Homoplastic frozen-dried sleeves provoke no marked inflammatory reaction. However, adhesions are not uncommon, their extent depending more on the condition of the wound bed than of the artery. Heteroplastic frozen-dried arteries (macaque in spider monkey) cause heavier adhesions than do homoplastic ones. Keeping rehydrated arteries for a few days refrigerated in Ringer's solution does not seem to harm them. Fresh arteries stored for several weeks in aqueous solution of Merthiolate 1:1000 and then washed, proved less adequate. Other methods of preservation (boiling, alcohol, formaldehyde) are definitely contraindicated, as they transform the artery into a foreign body and deprive it of many properties essential for nerve splicing. Veins tried in the monkey have proved too flabby for the purpose. No artificial substitute has as yet been found that would come up to the standards of the arterial sleeve; they all fall short of one or more of the principal functions of the sleeve, which are:[2] to align the nerve ends; to permit longitudinal stress to act on the "scar"; to prevent dissipation of fibrinolytic agents from the gap and thus insure proper liquidity of the "scar"; and to keep fibrous tissue and other local growth from penetrating. Continued research may yet produce a synthetic sleeve answering these demands.

For end-to-end reunion of stumps under tensions greater than an arterial cuff can hold, means must be devised to reconcile the need for forcible approximation with the cardinal precept for good regeneration, viz., to keep the zone of union free from artificial intervention, particularly sutures. A solution may lie in approximating the stumps by means of a permanent loop of Tantalum or Columbium wire, stitched either through the nerve stumps or the epineuria at considerable distance (more than one inch) from the cuts, and then splicing the free ends with an arterial sleeve just as in unstretched unions. It may be expected that the gradual "give" at the points of attachment will place enough strain on the young tissue connecting the stumps to produce in it the desired longitudinal organization. Results of this operation will be reported on a later occasion. Experiments are also under way to determine the maximum length of a blood filled gap between stumps compatible with optimal nerve regeneration.

Summary. Results obtained with sutureless nerve reunion by means of cuffs of live or frozen-dried artery in 21 rabbit, 30 cat, and 81 monkey nerve splices have confirmed for the larger mammals the conclusion previously reached in the rat, that optimal nerve regeneration may be secured by this method.

14402 P

Functional Nerve Regeneration Through Frozen-Dried Nerve Grafts in Cats and Monkeys.*

PAUL WEISS.

From the Department of Zoology, University of Chicago.

As reported previously,[1] grafts of frozen-dried and rehydrated nerve in the rat form excellent conductors for regenerating fibers. Extension of these experiments to cats and monkeys has given the following results. Six of 10 cat grafts and 21 of 36 spider monkey grafts have thus far been examined functionally.

Material and Methods. The nerves, either intact or predegenerated, were frozen in Isopentane at approximately −150°C, dehydrated over P_2O_5 *in vacuo* for one week, sealed, stored for from 2 to 4 months, and then rehydrated in Ringer's solution *in vacuo*. Segments of from 1 to 3 cm in length were used to bridge corresponding gaps in the tibial or peroneal nerve severed in the middle thigh. The grafts were joined to the stumps by sleeves of fresh or frozen-dried artery. Heteroplastic frozen-dried grafts (from macaque or cat to spider monkey) were used in some instances. Some grafts were not connected with the stumps immediately but first allowed several days of local conditioning at the site of the lesion. Occasionally multiple grafts were used, either in parallel or in tandem. Grafts must not be stripped of their perineuria.

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* This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago; also aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. The monkey experiments were carried out at the Yerkes Laboratories of Primate Biology, Orange Park, Florida, and I am greatly indebted to Dr. K. S. Lashley for his generous contribution of laboratory facilities. Technical assistance by Dr. R. W. Sperry and Dr. A. C. Taylor is also gratefully acknowledged.

[1] Weiss, P., and Taylor, A. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 326.

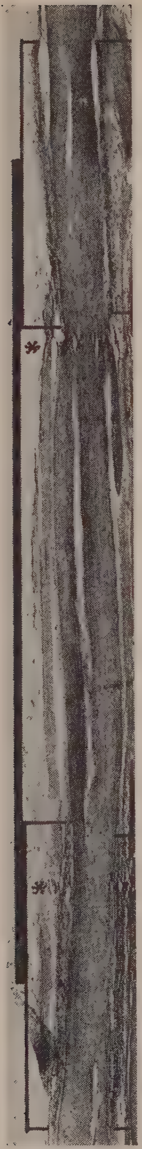

FIG. 1.
Cat tibial nerve 6 months after receiving frozen-dried graft. Bodian impregnation. ×7. Proximodistal direction from top to bottom. Splicing arteries are marked by brackets, the approximate extent of the graft (consisting of double nerve) is marked by a solid line. Asterisks indicate flaws due to histological sectioning. Note absence of "suture lines."

Results. A. Cat. Motor recovery of the peroneal nerve, as tested by dorsiflexor and toe-spreading reflexes, began at 3 months, and was nearly complete at 5-6 months after the operation. Fig. 1 illustrates full histological restitution of a tibial nerve through a 17-mm frozen-dried graft, 5 months p. op. (The peroneal nerve of this specimen had likewise received a graft.) Functional recovery had been clinically complete and the combined weight of the reinnervated leg muscles was only 6.5% below that of the intact control side. Fiber caliber and myelinization have well recovered at sample levels of 50 and 130 mm regeneration distance (Fig. 2).

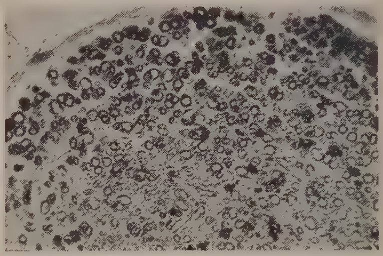

FIG. 2.
Cross section of regenerated portion of nerve of Fig. 1; 5 cm distal to graft. Osmic acid. × 120.

B. Monkey. Motor recovery was tested both by observation of spontaneous and reflex function and by electrical stimulation of the exposed nerves. Of 21 nerves examined between 5½ and 10 months after the operation, functional restoration was excellent in 8. good in 4, fair in 3, poor in 2 cases. Total failure in 4 cases has been accounted for by faults of technic. An interpretation of the variability of the results will be given in a later report in connection with the histological findings. Full recovery has occurred after the use of homoplastic grafts, as well as macaque-to-spider grafts, in the latter case with perceptible delay. Success with cat-to-monkey grafts was negligible. In a fully recovered homoplastic test case, electric shocks to the peroneal nerve proximal to the graft site gave strong contraction in intrinsic foot muscles, at 320 mm regeneration distance, when tested 182 days after the operation; this means that regeneration had proceeded at a minimum daily average of nearly 2 mm including the graft and its junctions.

No constant differences between predegenerated and non-predegenerated, or between conditioned and unconditioned, grafts have as yet been ascertained, although such differences might be expected in view of the fact that the "devitalized" grafts are being repopulated by host cells. As in nerve grafting in general, success depends on the proper way of joining the graft to the stumps. The method of sutureless sleeve-splicing[2] has been instrumental in securing the success of the frozen-dried grafts in the present experiments.

Summary. Full functional recovery may be obtained in cats and monkeys after the bridging of a nerve gap of several centimeters by grafts of frozen-dried nerve, stored for several months and rehydrated before use.

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[2] Weiss, P., Proc. Soc. Exp. Biol. and Med., 1943, preceding paper.

14403

Neurotropic Virus Infections in Chicago, 1939-1941. Nine Cases of Lymphocytic Choriomeningitis.

Albert Milzer. (Introduced by L. N. Katz.)

From the Samuel Deutsch Serum Center of Michael Reese Hospital, and Michael Reese Research Foundation, Chicago.

Ordinarily it is difficult or impossible to diagnose the various neurotropic virus infections of man on the basis of clinical or pathological findings because of their great similarity. For this reason, laboratory procedures must be employed for identification of the specific virus. During epidemics, it may be possible to identify such infections as poliomyelitis by means of characteristic findings. On the other hand, laboratory diagnosis becomes mandatory if two or more viruses are known to be endemic in the same region. Thus mixed epidemics of St. Louis encephalitis and western equine encephalomyelitis have been reported in certain of the Western states.[1-3] Laboratory aid is also essential for the diagnosis of lymphocytic choriomeningitis because of the protean clinical manifestations this virus produces and the fact that this infection is highly sporadic in man.[4]

The present studies were carried out in order to identify possible etiologic viruses in 75 patients suffering from various sporadic central nervous system infections in the Chicago area. In most instances, specimens of both spinal fluid and defibrinated blood obtained during the acute stage were routinely inoculated into Swiss mice and guinea pigs. Blood specimens were defibrinated by means of glass beads. In fatal cases, small portions of tissue obtained from different areas in the brain and cord were triturated in a mortar with sand, and a 10% emulsion in buffered saline (pH 7.4) was prepared. Most specimens were inoculated into animals within an hour after collection from the patient. Usually each specimen was inoculated intracerebrally (0.03 cc) into each of 6 mice and both intracerebrally (0.2 cc) and intraperitoneally (1 to 4 cc) into each of 2 guinea pigs. All animals remaining normal for 30 days after the original inoculation were tested for immunity to lymphocytic choriomeningitis by intracerebral inoculation with approximately 10 MLD of known virus. Bacteria were ruled out by routine inoculation of various aerobic and anaerobic culture mediums. Evidence that our stock mice and guinea pigs were free from latent infection with the choriomeningitis

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[1] Howitt, B. F., *Am. J. Pub. Health*, 1939, **29**, 1083.

[2] Meiklejohn, G., and Hammon, W. McD., *J. A. M. A.*, 1942, **118**, 961.

[3] Hammon, W. McD., and Howitt, B. F., *Am. J. Hyg.*, 1942, **35**, 163.

[4] Armstrong, C., *Harvey Lectures*, Lancaster, Pa., Science Press, 1941, 39.

TABLE I.
Summary of Virus Studies of Various Central Nervous System Infections of Man in Chicago, 1939-1941.

		Lymphocytic choriomeningitis			St. Louis encephalitis		Western equine encephalomyelitis	
Clinical diagnosis	No. of cases	Virus isolated	Positive neutralization test	Positive complement fix. test	Virus isolated	Positive neutralization test	Virus isolated	Positive neutralization test
Preparalytic poliomyelitis	28	1	1 (8 wks)	1 (8 wks)	—	0	0	0
Paralytic poliomyelitis	11	1	1 (9 ”)	1 (3 ”)	—	—	—	—
Acute encephalitis	20	3	3 (8 ”) (8 ”) (20 ”)	3 (3 ”) (3 ”) (12 ”)	0 (5 fatal)	1 (5 mos.)*	0	0
Aseptic meningitis	15†	2	2 (8 ”) (24 ”)	2 (4 ”) (24 ”)	—	0	0	0
“Influenzal” infection	1	1	1 (10 ”)	1 (10 ”)	—	0	0	0
Normal adults	10	0	0	0	—	0	—	0
Total	85	8	8	8	0	1	0	0

* Patient had a “sleeping episode” 5 years previously when living in San Diego, California.

† Three cases were positive for choriomeningitis—virus was isolated and antibodies were detected in one; virus was isolated from the second, but no convalescent serum could be obtained; in the third, no attempt was made to isolate virus but antibodies were demonstrated.

virus has been published elsewhere.[5] Moreover, no stock animals were encountered that were immune to inoculation with fatal amounts of virus.

Blood sera obtained during the acute stage of the disease and again several weeks or months after the attack were tested for the presence of neutralizing antibodies against the following viruses: (1) the W. E. (Rivers) or J. P. strain[6] of lymphocytic choriomeningitis, (2) the D.219 or Hubbard strain of St. Louis encephalitis, and (3) the B.A.I. strain of the western type of equine encephalomyelitis. The protection tests with the choriomeningitis virus were performed by inoculating guinea pigs subcutaneously with mixtures of equal parts of undiluted serum and serial tenfold virus dilutions according to the technic already described in connection with other studies.[5] Howitt's technic[1] was followed in protection tests with the St. Louis encephalitis and western equine encephalomyelitis virus. The sera were also tested for the presence of complement fixing antibodies against the choriomeningitis virus. Essentially the same procedure described by Smadel, Baird, and Wall[7] was used in the complement fixation test. The complement fixing antigen consisted of saline extracts of pooled infected guinea pig spleens. Known negative and positive control serums were included in all neutralization and complement fixation tests.

Results obtained in the virus and immunologic studies of 75 patients are summarized in Table I. All but one of these patients were examined and diagnosed by Drs. S. O. Levinson and A. M. Wolf, who are consultants on poliomyelitis to the Department of Public Health of the State of Illinois. As shown in Table I, 9 cases were positive for lymphocytic choriomeningitis, 2 of which were laboratory infections. Virus studies on the 11 cases of poliomyelitis showing paralysis were included because of atypical clinical findings which made the diagnosis questionable in the minds of the clinicians.

The pertinent clinical data on these patients are given in Table II. In 8 of the positive cases, no specific neutralizing or complement fixing antibodies were detected in the acute stage of the disease, but they subsequently

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[5] Milzer, A., *J. Infect. Dis.*, 1942, **70**, 152.

[6] Leichenger, H., Milzer, A., and Lack, H., *J. A. M. A.*, 1940, **115**, 436.

[7] Smadel, J. E., Baird, R. D., and Wall, M. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 71.

TABLE II.
Clinical Data on Nine Proven Cases of Lymphocytic Choriomeningitis in Chicago.

Clinical diagnosis	Patient	Age	Color and sex	Max. cell count spinal fluid	% lymphocytes	Date of onset
Preparalytic poliomyelitis	F.M.	10	W.F.	297	55	June 41
Paralytic ”	R.M.	14	W.M.	265	80	Aug. 40
Encephalitis	B.D.	22	W.F.	2	100	May 41
”	A.M.*	23	W.M.	8	100	Aug. 39
”	J.A.	2	W.M.	31	70	Sept. 41
Aseptic meningitis	J.P.	24	C.M.	970	90	Oct. 38
” ”	B.B.	19	W.F.	620	60	May 40
” ”	F.R.*	26	W.M.	—	—	May 41
“Influenzal” infection	A.V.	34	W.M.	—	—	Sept. 40

*Laboratory infections.

appeared during convalescence as indicated in parenthesis in Table I. Although convalescent serum was not obtained in the ninth case (J.P.), the diagnosis was established by the isolation of virus during the acute stage and the absence of specific antibodies. The choriomeningitis virus was isolated from spinal fluid or blood taken from 8 of the patients during the attack; in the remaining case (F.R.), no attempt was made to isolate virus, but the development of specific antibodies during convalescence was demonstrated. This last patient became infected in the laboratory following an accidental puncture of the skin of the leg with a hypodermic needle contaminated with infectious material. The second laboratory infection (patient A.M.) which occurred apparently resulted from contact with infected rhesus monkey lice. A detailed clinical report of this latter case and two other cases of the present series (J.P. and B.D.) have appeared in separate publications.[8,6,9]

Other studies which were made show that there is complete cross-immunity between the eight isolated strains of choriomeningitis virus and the W.E. (Rivers) strain as demonstrated by resistance to intracerebral inoculations in mice which have been infected and recovered. However, the pathogenicity of the isolated strains varied considerably with infectivity titers ranging from 10^{-4} to 10^{-7} dilutions. Microscopic examination of stained sections of the brains of guinea pigs and mice that succumbed to the various strains showed the round cell infiltration of the meninges and choroid plexus comparable to that produced by the W.E. strain.[10] All of the isolated strains suspended in nutrient broth of pH 7.2 (5% brain tissue suspension) and centrifuged at 2,000 r.p.m. to remove large tissue particles readily pass through previously tested Berkefeld N and W candles.

The protean clinical manifestations of lymphocytic choriomeningitis noted in the tables have also been reported by others.[4,11] A few established cases of paralysis, which were initially diagnosed as poliomyelitis, have occurred in England.[12,13] Several of the English patients developed complete paralysis of both lower extremities as well as facial and bladder paralysis. Our patient (R.M.) had paralysis of the lower abdominal muscles. Ordinarily, lymphocytic choriomeningitis infection is systemic in character and rarely involves the central nervous system. Therefore, it does not deserve serious consideration in the differential diagnosis of poliomyelitis. This is substantiated by Armstrong's finding[4] that approximately 11% of 2,000 serums taken at random from the general population contained neutralizing antibodies against the choriomeningitis virus, although only very few of the positives gave a history of any central nervous system infection. Furthermore, one

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[8] Milzer, A., and Levinson, S. O., *J. A. M. A.*, 1942, **120**, 27.

[9] Treusch, J. V., Milzer, A., and Levinson, S. O., *Ach. Int. Med.*, 1943, **72**, 709.

[10] Rivers, T. M., and Scott, T. F. McN., *J. Exp. Med.*, 1936, **63**, 415.

[11] Howard, M. E., *Yale J. Biol. and Med.*, 1940, **13**, 161.

[12] Findlay, G. M., Alcock, N. S., and Stern, R. O., *Lancet*, 1936, **1**, 650.

[13] MacCallum, F. O., and Findlay, G. M., *Lancet*, 1939, **1**, 1370.

of our patients (A.V.) had an "influenzal" infection without symptoms of central nervous system involvement. For this reason, no spinal tap was done, but the diagnosis was established by isolation of virus from the blood and the finding that specific choriomeningitis antibodies subsequently developed. This case was similar to the two described by Armstrong.[14,15]

Another point of interest is that 2 of our patients became ill in June and August (Table II). These cases are the first recorded for these months. All of the proven cases in the literature to date have occurred during the cooler portions of the year with the greatest incidence during October, November, and December.[15]

Tests for St. Louis encephalitis and western equine encephalitis gave negative results in all except one instance (Table I). This was one of the choriomeningitis patients (B.D.) who also had neutralizing antibodies for St. Louis encephalitis. This patient had suffered from a "sleeping episode" 5 years previously when she lived in a part of California where St. Louis encephalitis is known to be endemic.[16] The choriomeningitis virus was isolated from the spinal fluid on 2 occasions during her illness, and she subsequently developed an increased titer of both complement fixing and neutralizing antibodies for lymphocytic choriomeningitis. Further details of this case will appear in another publication.[9]

There are only two references in the literature to our knowledge concerning the presence of St. Louis encephalitis and western equine encephalomyelitis in Chicago. Wooley and Armstrong[17] reported that of 7 random sera collected in Chicago, 6 gave no protection and one gave questionable protection against the St. Louis encephalitis virus. On the other hand, 11 of 20 sera collected in Paris, Illinois, where the first cases of the 1933 St. Louis epidemic occurred, showed definite protection. More recently the occurrence of a sporadic case of western equine encephalomyelitis in a patient who had not been outside of metropolitan Chicago for many years has been described by Richter.[18] This author also tested the sera of 11 other patients with acute encephalitis in addition to twelve random controls for western equine encephalomyelitis neutralizing antibodies but obtained negative results.

Summary. Virus studies were carried out on 75 patients suffering from various sporadic central nervous system infections of unknown etiology. Nine cases of lymphocytic choriomeningitis proven by virus isolation or detection of specific antibodies are presented. The cases were clinically divisible as follows: (1) encephalomyelitic type, (2) meningeal type, and (3) an "influenzal" type without neurologic signs. Negative results were obtained in tests for St. Louis encephalitis and western equine encephalomyelitis.

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[14] Armstrong, C., *Pub. Health Rep.*, 1941, **56**, 907.

[15] Armstrong, C., *Mil. Surgeon*, 1942, **91**, 129.

[16] Howitt, B. F., *Am. J. Pub. Health*, 1942, **32**, 503.

[17] Wooley, J. G., and Armstrong, C., *Pub. Health Rep.*, 1934, **49**, 1495.

[18] Richter, R. B., *J. A. M. A.*, 1942, **119**, 486.

14404

Measurement of Toxicity of Antiseptics by the Method of Unstained Cell Counts.*

ROBERT SCHREK. (Introduced by Paul E. Steiner.)

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The method of unstained cell counts is a simple, quantitative, and direct means of determining the capacity of an agent to kill cells in suspension.[1] The method seemed, therefore, suitable for studying the toxicity of antiseptics. The present work consists of the measurement of the cytocidal activity of a few antiseptics.

Method. Suspensions of cells were prepared by mincing rabbit thymus and bone marrow with scissors and filtering through wire gauze as described in detail in a previous paper.[1] To 0.2 ml of the suspensions were added 3.8 ml of an eosin solution (1:1000 in Tyrode's fluid). A drop of the mixture was placed in a hemocytometer and the stained, unstained, and red blood cells were counted. The suspensions were then diluted with Tyrode's solution, when necessary, to give a standard count of approximately 200,000 unstained cells per cubic millimeter.

The following commercial preparations of antiseptics were used: zephiran chloride, aqueous and tincture, stainless, 1:1000; merthiolate, aqueous and tincture, 1:1000; metaphen, aqueous 1:500 and tincture 1:200; and phemerol, aqueous, 1:1000. The following stock solutions were prepared in distilled water: mercuric chloride 1:100, crystal violet 1:1000, phenol 1:20, and sodium sulfathiazole 1:50. All of these antiseptics were diluted with Tyrode's solution.

To 0.2 ml of the cell suspension was added an equal volume of diluted antiseptic so that the final concentrations of the reagents were 1:100, 1:200, 1:500, 1:1000, 1:2000, etc. The mixtures were shaken mechanically in a water bath at 37°C for 30 minutes. After incubation, 3.6 ml of the eosin solution were added and counts of the unstained, stained, and red blood cells were again made. From these results it was possible to estimate the concentration of antiseptic that would kill 90% of the original viable cells.

Observations. Various dilutions of the antiseptics tested were titrated against suspensions derived from rabbit thymus and bone marrow. The predominating nucleated cells in these two suspensions were the lymphocyte and the polymorphonuclear leukocyte respectively. The results are summarized in Table I which shows the concentrations of antiseptic that killed 90% of the unstained cells.

It is seen from this table that the reagents differed considerably in their cytocidal action. Sodium sulfathiazole was the least toxic; a dilution of 1:100 killed only 30 to 40% of the cells of the thymus and bone marrow. Mercuric chloride was the most toxic; a dilution of 1:30,000 sufficed to destroy the viability of 90% of the lymphocytes.

A second finding was that the tinctures of merthiolate and zephiran chloride were more toxic than the corresponding aqueous solutions. In the case of metaphen, the solution and the tincture were equal in toxicity.

All the antiseptics tested were found to be less toxic to the polymorphonuclear leukocyte than to the lymphocyte. This difference in toxicity was particularly marked with crystal violet; 90% of the cells of the suspension from the bone marrow were killed by a dilution of 1:2,400 of the dye, while the cells of the thymus were killed by a dilution of 1:16,000. The resistance of the polymorphonuclear leukocyte was also evidenced by its capacity to withstand the addition of the undiluted commercial antiseptics, merthiolate and phemerol in aqueous solution.

Discussion. Four *in vitro* methods have been developed for measuring the toxicity of

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* Published with the permission of the Medical Director of the Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.

[1] Schrek, R., *Arch. Path.*, 1943, **35**, 857.

TABLE I.
Concentrations of Antiseptics That Killed 90% of the Nucleated Cells in Suspensions Derived from Rabbit Thymus and Bone Marrow.

	Suspension from rabbit thymus		Suspension from rabbit bone marrow	
	Aqu. sol. of antiseptic	Tincture	Aqu. sol. of antiseptic	Tincture
Sodium sulfathiazole	(1:100)*		(1:100)*	
Phenol	1:260		1:300	
Mercurochrome	1:800		1:120	
Metaphen	1:7,000	1:6,000	1:2,400	1:2,800
Merthiolate	1:8,000	1:16,000	(1:2,000)*	1:8,000
Phemerol	1:12,000		(1:2,000)*	
Crystal violet	1:16,000		1:2,400	
Zephiran chloride	1:18,000	1:28,000	1:3,000	1:8,000
Mercuric chloride	1:30,000		1:3,800	

* Less than 90% of the cells were killed by the maximal concentration tested.

antiseptics on various physiologic functions of the animal cell. The tissue culture method utilized by Lambert[2], German,[3] Buchsbaum,[4] Salle,[5] Osgood,[6] Herrell,[7] and their associates studied the effect of the antiseptic on the growth and the microscopic appearance of cells. Bronfenbrenner, Hershey, and Doubly[8] compared the effect of reagents on the respiration of animal and bacterial cells by means of the manometric method. Nye,[9] Welch,[10] and Hirsch[11] and their co-workers utilized the inhibition of phagocytosis by polymorphonuclear leukocytes as a means of measuring the toxicity of antiseptics. Recently Witlin[12] and Dunham[13] have determined the effect of reagents on the viability of chick embryos.

There are, then, methods for studying the toxic effect of antiseptics on 3 physiologic functions, namely, the growth and respiration of cells and the phagocytosis by leukocytes. The method of the unstained cell count provides a means of determining the effect of antiseptics on a fourth important property of living cells, *i.e.*, their resistance to staining with eosin. Inasmuch as this resistance is a function of the viability of the cell and is rapidly lost when the cell "dies," the method permits the measurement of the cytocidal action of antiseptics.

The present work was undertaken to determine the value of the method of unstained cell counts for measuring the toxicity of antiseptics. The findings are not sufficient to demonstrate the superiority of one antiseptic to that of another.

One observation made in this and other studies seems of particular interest. With all the reagents tested in this and other experiments the polymorphonuclear leukocyte proved much more resistant than the lymphocyte and testicular cells. Since the polymorphonuclear leukocyte is a specialized type of cell, the main function of which is prompt protection against deleterious agents, it is not surprising to find that this cell is much more resistant to a wide variety of toxic agents.

The greater susceptibility of the lymphocyte to antiseptics makes this cell particularly useful in measuring the toxic properties of antiseptics.

Summary. The method of unstained cell counts provides a simple quantitative means of determining the toxicity of antiseptics to cells. The antiseptics tested differed markedly in their cytocidal activity, sodium sulfathiazole being the least and mercuric chloride the most toxic. Tinctures of merthiolate and zephiran were more toxic than the corresponding solutions. The polymorphonuclear leukocyte was more resistant to most of the reagents tested than the lymphocyte.

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[3] German, W. J., *Arch. Surg.*, 1929, **18**, 1920.

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[9] Nye, R. N., *J. A. M. A.*, 1937, **108**, 280.

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[13] Dunham, W. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 274.

14405

Direct Stimulation of the Hypoglossal Nucleus by Acetylcholine in Extreme Dilutions.

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Acetylcholine (ACh.), when applied in minute amounts to the eserinized cerebral cortex, induces marked excitatory effects;[1] it was inferred that the ACh. stimulates or facilitates various cortical synapses. The experiments here reported indicate that ACh., on direct application in extreme dilutions, stimulates a motor nucleus, namely the hypoglossal nucleus; this nucleus is readily accessible, as the *trigonum hypoglossi,* immediately beneath the floor of the fourth ventricle.[2]

Methods. Decerebration under ether was by the Sherrington decerebrator. Both common carotids were tied or, in other experiments, both internal carotids were tied and also the branches of external carotids, but leaving lingual arteries intact; essentially similar results were obtained in both preparations. The skull was held in a special clamp, so that the entire tongue could be observed. The floor of fourth ventricle was exposed by removing median parts of cerebellum. Acetylcholine chloride (ACh.Cl) (Merck) was dissolved in buffered Ringer-Locke solution and was applied in small rectangles 2 mm x 6 mm of spot test paper to the XII nuclei of both sides.

Results. Preliminary localization of effects from the XII nucleus was by unipolar faradization with a fine silver wire, ending in a minute bulb; the diffuse electrode was a saline-soaked pad on animal's back. Secondary distances varied from 35 to 50 cm, using a large Palmer inductorium (Fig. 1).

The following effects of ACh. were obtained in a decerebrate cat (3100 g), having lingual arteries intact. Preliminary eserinization was by 0.25 mg eserine sulphate (Merck) intravenously. A rectangle of spot test paper soaked in ACh.Cl. 1:50 millions was applied to both XII nuclei: in 4 sec. a slight median furrowing appeared at base of tongue between circumvallate papillæ (Fig. 2); the furrowing, which coincided with inspiration, increased and was accompanied by simultaneous retraction of tongue base, elevation of soft palate and dilatation of remainder of mouth cavity. The ACh. increased respirations from 22 to 32/min.; these observations on respiratory stimulation agree with those of Worzniak and Gesell[3] and of Hansen, Worzniak and Gesell.[4]

Application of another rectangle of ACh.Cl. 1:10 millions intensified above effects within 5 sec.; in consequence of a further application of a rectangle of ACh.Cl. 1:10 millions and 2 successive rectangles of ACh.Cl. 1:1 million, with 0.25 mg eserine intravenously, the following symptoms developed (Fig. 2): a marked furrowing at base of tongue, coin-

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[1] Miller, F. R., Stavraky, G. W., and Woonton, G. A., *J. Neurophysiol.*, 1940, **3**, 131.

[2] Winkler, C., and Potter, A., *An Anatomical Guide to Experimental Researches on the Cat's Brain,* W. Versluys, Amsterdam, 1914.

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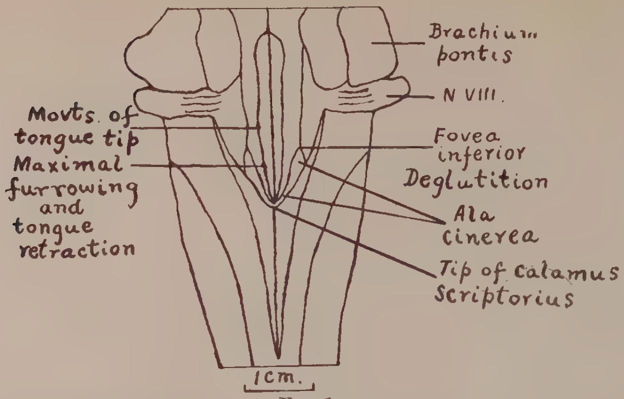

FIG. 1.

Floor of fourth ventricle of cat. × 2. (Wt of cat 3700g.) Effects of unipolar faradization: XII nucleus yielded movements of tongue tip at point shown; caudally to near tip of calamus scriptorius it yielded median longitudinal furrowing and general retraction of tongue; maximal furrowing at point shown. Inferior fovea yielded deglutition.

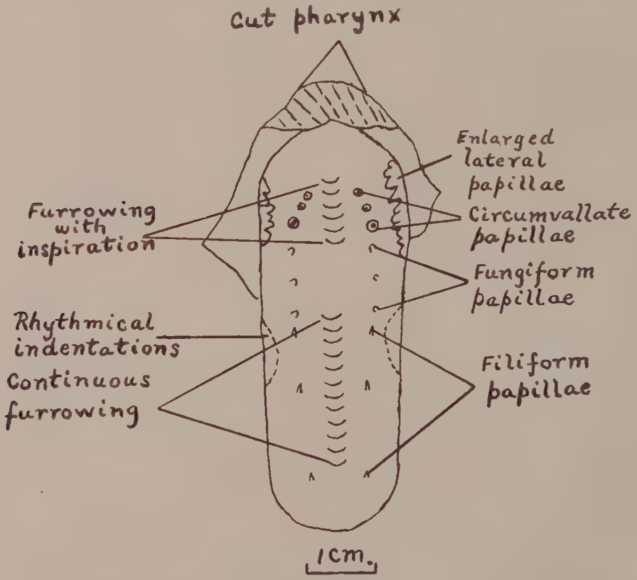

FIG. 2.

Tongue of cat; actual size. Effects of ACh. applications to XII nuclei. Rectangles of spot test paper 2 mm × 6 mm soaked in ACh. were applied from point for tongue tip back to calamus (Fig. 1). Dilutions of ACh. 1:50 millions, later 1:10 millions and 1:1 million. Cat eserinized.

cident with inspiration, also simultaneous elevation of soft palate and dilatation of mouth cavity, as at first, but all more intense; a continuous furrowing farther forward, with bilateral rhythmical indentations; all these movements were very violent; at the same time respirations were accelerated to 40/min.

Four mg atropine sulphate (Merck) were then given intravenously, whereupon, within 15 to 20 sec., all the above tongue and other movements ceased completely, while respirations fell to 22/min. Repetition of intravenous eserine and application to the XII nuclei of ACh.Cl. 1:1 million now yielded none of the previous effects on tongue, palate or mouth cavity, while the respiratory rate remained unaltered. Thus atropinization abolishes and precludes the action of ACh.

In another experiment a rectangle of ACh.Cl. 1:50 millions to the XII nuclei, followed by one of ACh.Cl. 1:10 millions augmented tremors in the tongue, initiated by intravenous eserine, and also evoked furrowing, tongue retraction and later violent generalized tongue movements; applications also evoked repeated deglutitions.

In a *non-eserinized* decerebrate preparation ACh.Cl. 1:50 millions on both XII nuclei yielded, in 35 sec. (longer than after eserinization), movements of tip of tongue, retractions and repeated deglutitions; thus, while responding to ACh., the nucleus was definitely less sensitive than after eserinization; also its responsiveness declined to further applications.

Discussion. The view is expressed here that ACh. exerts a local stimulating or facilitating action on synaptic terminations in the XII nucleus; support for this view is afforded by the following considerations: the general similarity between effects of ACh. application and faradization of nucleus; the slight depth of nucleus below floor of fourth ventricle, permitting rapid diffusion to it of ACh., the extreme dilutions (1:50 millions) of effective solutions of ACh.; the brief latency for effects of ACh. (4 to 15 sec. in some cases). The neurones of the XII nucleus are surrounded by numerous synapses derived from an extensive interneuronal plexus.[5] Thus it is inferred that ACh. diffuses and directly stimulates or facilitates these synaptic terminations and that this action is much intensified by eserinization. The specific nature of the action is further emphasized through its being annulled by atropinization.

Deglutition may be explained by the action of ACh. near the inferior fovea; faradization of this point yields reflex deglutition[6] and it was supposed that the currents impinged on afferent fibers in the *tractus solitarius,* which lies sufficiently close to the inferior fovea. Similarly the ACh. may be supposed to diffuse to, and stimulate or facilitate, synaptic afferent terminations (probably glossopharyngeal and vagal) within the nucleus of the *tractus solitarius;* fibers from this nucleus probably pass to the XII nucleus, the nucleus ambiguus and dorsal vagus nucleus, through which pathways deglutition would be evoked.

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[5] Cajal, S. R., *Système Nerveux,* A. Maloine, Paris, 1909, **1**, 706.

[6] Miller, F. R., and Sherrington, C. S., *Quart. J. Exp. Physiol.,* 1916, **9**, 147.

14406

Production of Diabetes Mellitus in Rats with Alloxan.*

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Jacobs[1] observed that alloxan causes hyperglycemia followed by hypoglycemia in rabbits. Shaw Dunn and associates[2,3] have shown that the pathologic substrate of this response is necrosis of the pancreatic islets. Further study of the animals was impossible because they all died within a few days. Brunschwig, Allen, Goldner, and Gomori[4] succeeded in keeping rabbits alive for longer periods after

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* This work has been supported by grants from the Douglas Smith Foundation for Medical Research of the University of Chicago and from Eli Lilly and Company, Indianapolis.

[1] Jacobs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 407.

[2] Shaw Dunn, J., Sheehan, H. L., and McLetchie, N. G. B., *Lancet,* 1943, **244**, 484.

[3] Shaw Dunn, J., Kirkpatrick, J., McLetchie, N. G. B., and Telfer, S. V., *J. Path. and Bact.,* 1943, **55**, 245.

[4] Brunschwig, A., Allen, J. G., Goldner, M. G., and Gomori, G., *J. A. M. A.,* 1943, **122**, 966.

the injection of alloxan and found that the hypoglycemic phase is followed by temporary hyperglycemia. Bailey and Bailey[5] produced permanent diabetes in rabbits with the same chemical. Their histologic findings were complete disappearance of the islets. Goldner and Gomori[6] made dogs permanently diabetic by a single intravenous injection of alloxan. They observed degranulation and later complete disappearance of the beta cells while the alpha cells were found to be unaffected. Very recently, Shaw Dunn and McLetchie[7] reported the successful production of diabetes in rats. The histologic changes in the islets were necrosis of the central cells with survival of the peripheral cells. The latter were considered alpha cells although no special stains were applied to confirm this contention.

The experiments here described were performed independently of those of Shaw Dunn and McLetchie, after previous studies[6] had shown that rats are sensitive to alloxan.

Methods. Forty-four young white rats and 5 hooded rats, weighing 140 to 180 g, were used. It was found impossible to produce clinical symptoms by the subcutaneous injection of daily doses increasing from 50 to 175 mg/kg over a period of 8 days. Since, on the other hand, an intraperitoneal injection of 200 mg/kg was almost invariably effective, 40 rats were treated in this way.

A single dose of alloxan was injected intraperitoneally. A freshly prepared 5% solution in distilled water was used. Six animals were put in individual metabolism cages on the first day and killed by decapitation at intervals from the third hour on. Three or 4 more animals were placed in metabolism cages on each of the following 5 days. Their urine was collected for 24 hours and analyzed for sugar and ketone bodies. At the end of this period the animals were weighed and killed. The blood was analyzed for sugar only, since its amount was not sufficient for more detailed studies. The pancreas was fixed in a modified Bouin's solution (4 g of potassium chromium sulfate dissolved in a mixture of acetic acid, 5 cc; 40% formaldehyde, 25 cc; saturated aqueous solution of picric acid, 75 cc) which was found to permit a better differentiation of the cell types in the islets than other fixatives. The liver and the kidneys were fixed in 80% alcohol. All tissues were embedded in paraffin. Sections of the pancreas were stained with chrome hematoxylin and phloxin,[8] with Mallory-Heidenhain's azan stain and with Mallory's phosphotungstic hematoxylin as modified by Gomori.[8] Sections of the liver and kidneys were stained for glycogen with the Bauer-Feulgen[9] stain.

Results. Three white rats were found dead and cold in their cages and were discarded. In 5 animals no blood sugar determination was done. The 5 hooded rats were found to be resistant to alloxan under the conditions of these experiments. Fig. 1 shows the chemical findings in the remaining 27 animals and in 9 normal controls. Only 4 animals of this group were found to be resistant to alloxan. All the others showed hyperglycemia and glycosuria. Ketonuria was observed from the third day on and was present on subsequent days in all animals which had a blood sugar level above 300. Considerable polyuria was quite common; the daily amount of the urine often exceeded 10% of the body weight and reached 40 cc in one instance. Some of the animals had a pink urine on the first day after the injection. Many of the animals looked quite sick, especially after the first 2 days, but ate well until ketonuria set in with complete loss of appetite. They lost an average of 30% of their original weight in 6 days.

Histology. The following description applies to sections stained with chrome hematoxylin and phloxin.

Some nuclear pyknosis of the beta cells was observed as early as 3 hours after the injection. The 8 hours' specimen showed marked pyknosis of most of the beta nuclei with shrinkage of the cytoplasm. At 20 hours the overwhelming majority of the beta cells were greatly shrunken, and in some cases the cell cords were disintegrated into individual cells; in

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[5] Bailey, C. C., and Bailey, O. T., *J. A. M. A.*, 1943, **122**, 1165.

[6] Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

[7] Shaw Dunn, J., and McLetchie, N. G. B., *Lancet*, 1943, **245**, 384.

[8] Gomori, G., *Am. J. Path.*, 1941, **17**, 395.

[9] Bauer, H., *Z. mikr.-anat. Forsch.*, 1933, **33**, 143.

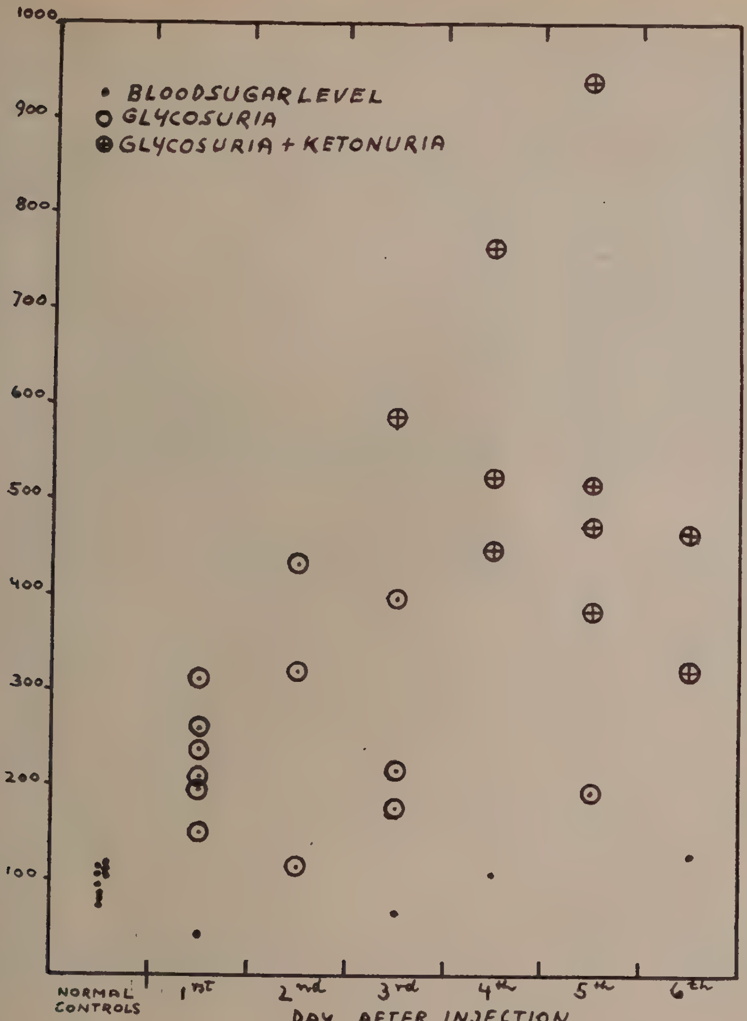

FIG. 1.

Hyperglycemia, glycosuria, and ketonuria in 27 rats after intraperitoneal injection of alloxan (200 mg/kg).

other cases the cords coalesced into an almost homogeneous debris in which individual cells could not be recognized. On the second day these changes became more accentuated, and, at the same time, the beta cells started to lose the typical staining of their granules and to be transformed into pale bluish-gray shadows. From the third day on these shadows disappeared very rapidly. On the fourth and fifth days only an occasional beta cell could be seen. At the same time agranular cells of an unidentifiable type appeared in large numbers and filled the areas previously occupied by the necrotic beta cells. At the periphery the alpha cells, which seemed to have escaped all injury, also proliferated considerably. By the fifth and sixth days the islets were entirely free from debris, and with the routine hematoxylin-eosin stain looked practically normal. However, special stains showed that they consisted of agranular and alpha cells only; beta cells were completely absent. No vacuolation

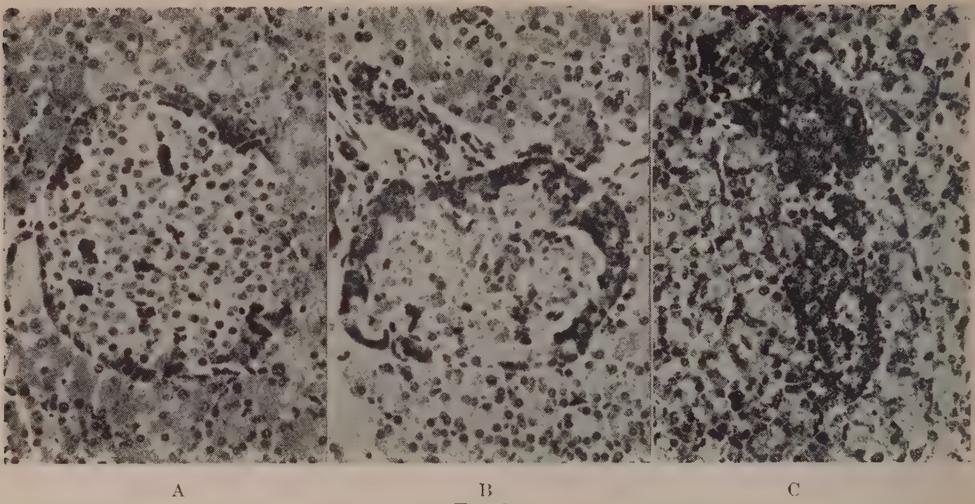

FIG. 2.

Microphotographs of pancreatic islets of rats (phosphotungstic acid hematoxylin stain).

A. (Left) Normal islet. Dark (alpha) cells at the periphery.

B. (Center) Necrosis of the central light (beta) cells on the second day. Dark peripheral (alpha) cells normal.

C. (Right) Sixth day. The islets consist almost exclusively of dark (alpha) cells.

of the islet cells was seen at any stage of the process; mitoses were extremely rare. The ducts and the acinar parenchyma showed no change.

The liver contained very little glycogen after the second day but was normal otherwise in all cases except 2 in which focal necroses were observed.

The kidneys did not show any change of importance. From the fourth day on deposition of glycogen in Henle's loops was quite common.

The organs of the hooded rats were completely normal. Out of the 4 white rats that failed to respond 2 showed no changes at all, while the 2 others showed slight degenerative changes of the beta cells.

Comment. Shaw Dunn and McLetchie produced diabetes in rats by repeated subcutaneous injections of alloxan; glycosuria usually became apparent after a number of injections had been given. In the present experiments a single intraperitoneal injection of alloxan was promptly followed by symptoms of diabetes. The histologic changes of the pancreas, as far as it is possible to compare the results obtained with different staining techniques, seem to be identical in the 2 experiments.

The remarkable resistance of hooded rats to alloxan may be due to the exceptionally large amount of islet tissue this strain possesses (Richardson and Young).[10]

Summary. A single intraperitoneal injection of 200 mg/kg of alloxan into white rats causes typical diabetes mellitus. The histologic changes are necrosis and complete disappearance of the beta cells from the pancreatic islets.

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[10] Richardson, K. C., and Young, F. G., *J. Physiol.*, 1937, **91**, 352.

14407 P

A Simple Technic for Studying the Periodicity and Survival of the Respiratory Center.

W. A. Selle.

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It was previously reported[1] that the isolated unperfused head of the young animal, unlike that of the adult, continues to gasp for some minutes following cessation of the cerebral blood flow and that the duration of gasping, as well as the total number of gasps, is inversely proportional to age for animals up to or slightly past the period of weaning. Thus, young rats (under 5 or 6 weeks of age) display two periods of gasping movements: an initial or aerobic series (I) of 6 to 12 gasps which lasts for a period of 15-40 seconds; and a second or anaerobic series (II) which begins 20-50 seconds after cessation of the initial series and lasts for a variable time depending upon age. This second series continues for 25-30 minutes in the newborn rat, 12-16 minutes in the 7-day-old animal, 4-7 minutes in the 14-day-old, 2-3 minutes in the 21-day-old and 1¼-1¾ minutes in the 28-day-old. Rats older than 5 or 6 weeks do not show a second series. Similar observations have been made on the ischemic heads of rabbits, cats, and dogs.[2]

Using 12-15-day-old rats (18-25 g) as test animals, it can be shown that various chemical agents administered to the animal prior to decapitation influence greatly the duration, frequency and character of gasps of either the initial or second series, or both. Chemical agents so tested are usually injected subcutaneously or intraperitoneally. In some instances they are given by mouth, or if volatile, as the general anesthetics, they are inspired. Following an interval ranging from 5 minutes to several hours or even days, depending upon the rapidity of action of the test material, the head is quickly isolated. The gasps resulting are mechanically recorded by use of a few simple pieces of physiological apparatus, such as a small burette clamp for holding the head and a light heart lever to which the mandible is attached by means of a bent pin. Action potentials of the brain stem can also be recorded readily with suitable equipment.

Since the survival time, as well as the total number of gasps, is influenced by litter or family characteristics, which in turn are modified by the state of development, the nutritive condition and the degree of hydration of the animal, it is important that the tests be carried out on litter mates subjected to identical cage conditions. Untreated litter mates of 14 days of age yield results which are remarkably uniform. Such animals show a respiratory pattern of 25-30 gasps following the onset of anoxia, there being a relatively constant number of gasps (about 8) in the initial series and a slightly variable number (17-22) in the second. The standard deviation of the mean number of gasps for this age group is slightly less than 2. In a series of preliminary experiments on 35 litters comprising 244 animals, the average variation in the survival time of untreated litter mates was found to be 8.4%; the average variation in the number of gasps of the second series was 6.1%.

The results for the test animals are compared with those of the litter mate controls given an amount of vehicle (usually 0.9% NaCl) equal to that used in the treated animals. Some drugs greatly increase the frequency and number of gasps and the duration (survival) of gasping; others diminish survival and alter the number and character of the gasps. Deviations from control values can be conveniently expressed in per cent.

With this relatively simple preparation and procedure, the following, which will be reported later in detail, can be demonstrated: (1) Survival of the respiratory center varies with blood glucose and glucose, lactate and pyruvate can counteract insulin; (2) the initial aerobic gasps are inhibited by minimal amounts of cyanide (0.1 cc of 0.01M) without alteration of the anaerobic ones; while (3) iodoacetic acid (0.1 cc of 0.01M) has the reverse action; (4) survival of the center is shortened by dinitrophenol, thyroxin, and other agents increasing metabolism; (5) gasping is depressed by most hypnotics and general anesthetics but may be stimulated by suitable doses of alcohol, morphine and chloralose; and (6) metrazol counteracts barbiturate depression.

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[1] Selle, W. A., and Witten, T. A., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 495.

[2] Selle, W. A., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 417.

The influence of temperature on the frequency and duration (survival) of the periodic discharges of the respiratory center can be dramatically demonstrated in 10-day-old animals (or younger) by immersing the isolated head in water of 20° and 40°C. If the area over the brain stem is quickly cooled with ethyl chloride spray, gasping is abruptly suspended; upon warming, gasping is resumed and a more or less normal series ensues. Heads of 1- to 4-day-old rats have been "revived" for a short period of activity after more than 50 minutes of cooling.

Summary. A method for studying the periodicity and survival of the primitive respiratory mechanism of young animals is described. Certain chemical and physical factors capable of influencing frequency, duration, and pattern of these periodic movements are indicated.

14408

Extensive Insulin Tolerance in Diabetic Dogs.*

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Soon after the introduction of insulin into clinical practice, it was observed that the amount of the new hormone required to control the glycosuria in patients with diabetes mellitus depended in part on the amount of available carbohydrate in the diet. Elaborate attempts were made to establish a quantitative relation between the insulin requirement of the patient and the amount of carbohydrate obtained as such in the food together with that which might arise through the conversion of protein and fat. Some success attended these efforts. It was soon found however that wide variations in the insulin requirement of certain patients occurred from time to time in spite of a constant diet and that enormous amounts of insulin were needed for certain patients.[1] The concept of insulin sensitivity and of insulin resistance thus arose. The precise nature of these states seems to be little understood and the present report is presented in the hope that it may contribute to the solution of this problem.

In a recent monograph, Jensen[2] states that about one unit of insulin per kilogram of body weight is required per day to maintain normal carbohydrate metabolism in the depancreatized dog. In our experience this figure is too low. Most of these animals require from 2 to 3 units per kg if good control of the glycosuria is desired. In comparison with these figures the amounts of insulin required to establish a comparable control of glycosuria in dogs with certain types of partial pancreatectomy seem surprising.

Nine male dogs, varying in weight from 8.2

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* This work has been aided by grants from the Josiah Macy, Jr., Foundation, the Douglas Smith Foundation for Medical Research of The University of Chicago, and Armour and Company. The insulin used was furnished by the Eli Lilly Company.

[1] Glass, W. I., Spingarn, C. L., and Pollack, H., *Arch. Int. Med.*, 1942, **70**, 221.

[2] Jensen, H. F., *Insulin*, The Commonwealth Fund, New York, 1938.

TABLE I.
The Insulin Requirement of Dogs After Partial and Complete Pancreatectomy.

Complete pancreatectomy				
Dog No.	Wt, kg	Days after operation	Remarks	Insulin units
D 495	7.7	0	Pancreatectomy	0
		7		6
		13		16
		30		26
		45		24
D 464	5.2	0	,,	0
		20		10
		30		8
		40		4
		60		4
		73		2
D 519	10.3	0	,,	0
		10		10
		14		28
		20		30
		60		22
		75		30
D 533	9.1	0	,,	0
		5		12
		10		20
		15		38
		20		44
		30		34
D 388	10.2	0	,,	0
		5		12
		15		20
		30		24
		40		26
		50		20
		60		18
		70		12
		80		10
		90		6
D 390	8.4	0	,,	0
		10		8
		20		8
		30		10
		40		16
		50		10
		60		2
D 393	8.3	0	,,	0
		5		14
		15		22
		25		24
		35		18
		45		12
		55		22
		100		20
		160		16
		180		8
D 445	6.6	0	,,	0
		10		20
		30		28
		40		26
		50		24
		60		26
		70		20
		80		16
		90		8

Partial Pancreatectomy				
Dog No.	Wt. kg	Days after operation	Remarks	Insulin units
D 387	10	0	17 g pancreas removed	0
		34	1.2 g left	20
		180		48
		240		60
D 373	13.4	0	23 g pancreas removed	0
		12	1.4 g left	20
		26		44
		41		72
		120		134
		210		152
		360		128
D 374	12.3	0	1 g pancreas left	0
		8		14
		30		6
		120		14
		150		44
		210		50
D 431	10.3	0	1.2 g pancreas left	0
		10		8
		20		40
		30		82
		60		50
		120		40
		240		30
D 156	8.2	0	1.5 g pancreas left	0
		10		3
		20		20
		40		40
		210		36
		360		40
D 430	10.5	0	1.17 g pancreas left	0
		10		8
		20		40
		30		96
		60		80
		120		80
		150		105
D 391	9.6	0	1.5 g pancreas left	0
		10		8
		20		32
		30		50
		40		58
		60		50
		90		54
D 392	8.8	0	1.5 g pancreas left	0
		5		8
		15		24
		25		36
		35		60
		45		70
D 386	12.2	0	2.0 g pancreas left	0
		10		12
		20		26
		30		44
		40		52
		60		54

to 13.4 kg, were operated upon and all of the pancreas removed save for a small portion in the region of the lesser duct. Patterns of pancreas tissue cut to the size of the remnants left weighed from 1 to 2 g so that the pancreatectomy was 90 to 94% complete. The diet of all the animals was similar and consisted of 600 g meat, 100 g bread, 400 cc milk, 25 g suet, 25 g bone meal, and 10 g brewer's yeast. This was fed in 2 meals and insulin was given after each feeding. The urine was examined daily for sugar and the dose of insulin adjusted to limit the glycosuria to less than 1 to 2 g per day. The insulin requirement of these animals is given in Table I together with a representative series of animals with total pancreatectomy for comparison. It is evident that dogs with partial pancreatectomy require definitely more insulin than after complete pancreatectomy and that occasionally animals may be found such as D 373 and D 430 where exceptionally large doses are tolerated. These animals were given 11 and 10 units of regular insulin per kg of body weight per day for long periods. This would correspond to 700 units for a 70-kg man and comes within the range of so-called insulin resistance.

Young[3] found that dogs made permanently diabetic by injections of anterior pituitary extracts also required more insulin than depancreatized animals on the same diet. At the present time we have two dogs in the laboratory made diabetic by the intravenous injection of alloxan and these require 32 and 36 units of regular insulin daily which is perhaps somewhat greater than for depancreatized animals at the same period.

It is likely that the somewhat better digestion and absorption in the animals with pancreas remnants is a factor in their greater insulin tolerance but this is probably not the complete explanation. The phenomenon recalls the greater tolerance of normal animals to the administration of thyroxin as compared with the same animals after thyroidectomy.

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[3] Young, F. G., *New England J. Med.*, 1939, **221**, 635.

14409

Effect of Tocopherols and Soybean Phosphatides on Utilization of Carotene.

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Slanetz and Scharf[1] have reported recently that soybean phosphatides have a sparing action on carotene and vitamin A. Rats fed a limited quantity of A-vitamins grew faster when soybean phosphatides were fed simultaneously. If this enhanced growth were due to the phosphatides in the preparation fed, it would reveal a new and important factor for the better utilization of vitamin A. At present, the only natural factors known to possess the power of vitamin A synergy are the tocopherols, either singly or as members of a covitamin E complex.

It occurred to us that the synergy observed in Slanetz and Scharf's experiment may have been due to the tocopherols contained in the crude soybean phosphatides employed. Calculations followed by analysis of a similar phosphatide product suggested that the soybean oil or the crude phosphatides in their experiment each contributed about the optimum daily supplement of tocopherols and would thus provide sufficient explanation for the synergism observed.

Experiments now to be reported in which the phosphatides have been treated for removal of tocopherols, indicate that the precipitated phosphatides are substantially inactive. At the same time, the materials removed from the phosphatides and established

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* Communication No. 55. Covitamin Studies, Part IV.

[1] Slanetz, Charles A., and Scharf, Albert, PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 17.

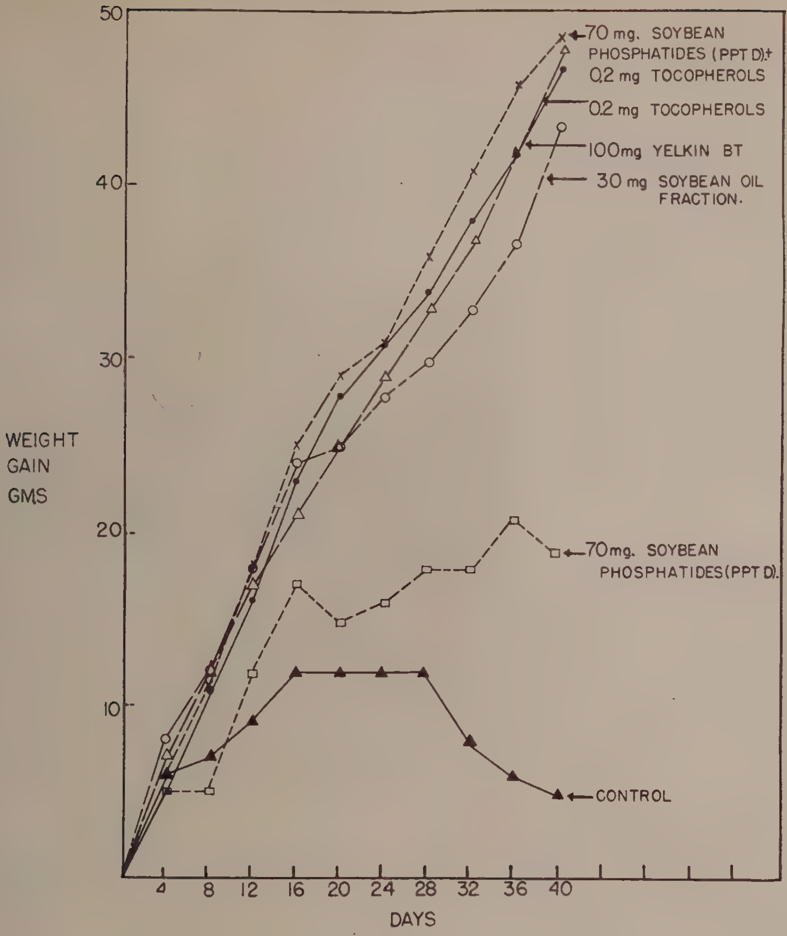

FIG. 1.

Growth of vitamin A depleted rats fed 1.2 μg carotene per day with and without protective agents.

to contain tocopherols are found to give the same growth enhancement as the crude phosphatides or natural mixed tocopherols.

Experimental. The diet used throughout this experiment contained casein (vitamin A-free), 18%; salt mixture (U.S.P. No. 2), 4%; yeast (dried Anheuser-Busch Strain G), 8%; starch, 30%; Cerelose, 35%; lard, 5%. The vitamin D content was 3 units per gram of diet supplied by activated ergosterol, A.R.P.I. Process. This diet was known to be very low in vitamin A and vitamin E potency.

Young male rats were depleted of vitamin A on this diet in the usual manner. At depletion, groups of 10 rats each were given 1.2 μg carotene (S.M.A. Corp.: 90% β + 10% α) daily together with supplements of soybean phosphatides and tocopherols, as listed in Fig. 1. The crude soybean phosphatide, Yelkin BT, was obtained from the same source[†] used by Slanetz and Scharf.

Preparation of Pure Phosphatides. Yelkin BT was dissolved in ether and the phosphatides precipitated by acetone. The treatment was repeated and the precipitate transferred to olive oil. The oil, containing a known weight of the precipitated phosphatides, was fed to the rats from a calibrated dropper.

[†] American Lecithin Company, Elmhurst, Long Island. This product contains about 30% soybean oil.

The ether-acetone extract of the crude phosphatides, hereafter referred to as soybean oil fraction was concentrated by evaporation to remove the ether and acetone. The concentrate was fed as such to one group of depleted rats.

The amounts of tocopherols present in the crude phosphatide and fractions were determined by the Emmerie and Engel[2] method and results are given in Table I.

TABLE I.
Tocopherol Content of Soybean Phosphatides by Chemical Analysis (Emmerie and Engel).

Material analyzed	Tocopherol content
	%
Yelkin BT (whole)	0.2
Soybean Oil Fraction	0.6
Precipitated phosphatides	0.01

Thus, all groups of rats except the control and one of the groups fed 70 mg precipitated phosphatides received 0.2 mg of tocopherols daily.

Discussion. It is evident from Fig. 1 that of the agents studied the tocopherols were chiefly responsible for the increase in weight gains over that of the control group. The rats fed 0.2 mg tocopherols per day showed weight gains as great as the rats fed 30 mg of the soybean oil fraction or 100 mg Yelkin BT. Both fractions contained 0.2 mg tocopherols by analysis. The addition of 70 mg precipitated phosphatides to the 0.2 mg tocopherol supplement did not improve the protective power of the tocopherols. However, the supplement of 70 mg of precipitated phosphatides was responsible for maintaining early growth gains after 4 weeks at a time when the rats receiving 1.2 μg carotene alone began to lose weight. This might be explained by the small quantity of tocopherols (0.01%) which survived the two precipitations of the phosphatides.

Slanetz and Scharf reported cessation in growth after three weeks of the rats fed soybean oil, but continued growth of the rats fed commercial soybean phosphatides. This observation may be explained in two ways. Either the soybean oil did not contain as much tocopherol as was present in the soybean phosphatides or their diet was deficient in factors which were present in soybean phosphatides but not in the soybean oil. The inability of rats fed 2 I.U. carotene to grow puzzled these workers. They suggested that their supplement of the B-vitamins, namely, thiamine, riboflavin, pyridoxine, niacin, choline, and pantothenic acid, was inadequate and that probably an unknown factor is present in soybean phosphatides, soybean oil, and yeast which is essential for the utilization of vitamin A and carotene and, furthermore, that this factor is not present in cottonseed oil. Cottonseed oil, presumably refined, constituted 4% of their diet. If the rats ate 7 g of the ration per day and the cottonseed oil contained 0.04% vitamin E, the rats would have received only about 0.1 mg of vitamin E per day. Previous work[3] has shown this amount to be borderline or inadequate for maximum growth when low levels of carotene were fed. Thus, it is probable that additional quantities of tocopherols would have resulted in growth of the animals fed the low level of carotene.

Slanetz and Scharf stated that "Even more striking was the fact that the animals receiving phosphatides were in much better condition at the end of the 4-week test period than the rats receiving only soybean oil. The fur of the soybean oil rats appeared shaggy and dry while that of the phosphatides rats was sleek and glossy." Comparison of our rats fed tocopherols with those fed Yelkin BT, soybean oil fraction, or soybean phosphatides plus tocopherols failed to show any difference in body condition or appearance of the fur between these groups.

The able assistance of Miss Joann Beers in managing the experimental animals is acknowledged.

Summary. The vitamin A-enhancing property of crude soybean phosphatides is due principally to the tocopherols present in these phosphatides.

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[2] Emmerie, A., and Engel, C., *Rec. trav. chim.*, 1938, **57**, 1351.

[3] Hickman, K. C. D., Harris, P. L., and Woodside, Marian, *Nature*, 1942, **150**, 91.

14410 P

Volumes of Basal Ganglia and Cortex in Mammals.

Pinckney J. Harman. (Introduced by W. C. Hess.)

From the Georgetown University Brain Research Institute, Washington, D.C.

A resurgence of interest in the basal ganglia has accompanied the recent excellent studies on these nuclei.[1] It is perhaps surprising, therefore, that the comparative morphology of the striatum and the pallidum has received so little attention. The present report presents data on the absolute volumes of these areas in a closely graded series of primates and in one sub-primate brain. Because of the linking of the basal ganglia with the cortex and the general problem of the relationship of brain size to development, the volumes of the cortex and the brain have been included in this study.

The method followed is that suggested by Dornfeld, Slater and Scheffe,[2] wherein the total volume is estimated by constructing a profile from the areas of sample cross sections (measured by planimeter) throughout the extent of the structure studied. In the case of the basal nuclei approximately every 20th or 40th section was measured, and in the case of the cortex and the brain approximately every 100th section was taken. All measurements were made on one side of the brain only (usually the right), hence the figures given in Table I are half-values for the total brain. The measurements in this series were made on preparations stained for fibers and do not take into account variations of cellular density which may be quite marked, particularly in the case of the pallidum.

The brains selected consist of one sub-primate, the sheep (Ovis); 2 primitive primates, Galago and Perodicticus (potto); 2 monkeys, Cebus and Cercocebus, comparable in development to the often studied rhesus monkey, *Macaca mulatta;* and the chimpanzee* (Pan).

Relative size is indicated in Table II where the volume of each gray mass is expressed in percentage of the total brain volume.

A consideration of the data presented reveals that in general the size of all the masses studied is roughly proportionate to the total brain size. This follows from the fact that the percentages for any one nucleus do not vary widely. Despite this, there is a change in the relationship of the caudate-putamen complex as one ascends the phylogenetic scale. Thus, the caudate nucleus is considerably larger than the putamen in sheep (Ovis) and Galago, slighter larger in Perodicticus (potto), and smaller in the remainder of the series.

TABLE I.

Volumes in Cubic Millimeters of Basal Ganglia, Cortex, and Brain of a Series of Mammals. N.B. These are half-values; *i.e.*, computed from one side of the brain.

			Pallidum			
	Caudate	Putamen	Lateral Seg.	Medial Seg.	Cortex	Brain
Ovis	364.3	210.5	61.9		10,165	20,485
Galago	18.9	14.9	2.8	1.1	644	1,346
Perodicticus	73.7	70.6	16.5	9.5	1,430	3,210
Cebus	280.6	490.8	67.3	37.4	10,530	19,100
Cercocebus	501.8	576.9	122.8	71.3	15,738	31,444
Pan	995.6	1,539.0	204.6	115.3	45,999	92,883

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[1] *The Diseases of the Basal Ganglia,* Research Pub. Assn. Res. in Nerv. and Ment. Dis., 1942, **21**.

[2] Dornfeld, E. J., Slater, D. W., and Scheffe, H., *Anat. Rec.,* 1942, **82**, 255.

* The writer is indebted to Professor Othmar Solnitzky, Director of the Georgetown University Brain Research Institute for making this material available, and to Professor J. F. Fulton and Dr. Margaret A. Kennard of Yale University School of Medicine, who donated several of the specimens to the Institute.

TABLE II.
Volume of Each Gray Mass Expressed in Percentage of Total Brain Volume.

	Caudate	Putamen	Pallidum Lateral Seg.	Pallidum Medial Seg.	Cortex
Ovis	1.8	1.0	.30		50.
Galago	1.4	1.1	.21	.082	48.
Perodicticus	2.3	2.2	.51	.30	45.
Cebus	1.5	2.6	.35	.20	55.
Cercocebus	1.6	1.8	.39	.23	50.
Pan	1.1	1.7	.22	.12	50.

The emergence of the putamen in Cebus, Cercocebus, and Pan as the most prominent nucleus of the basal group is a striking result of this study. According to Dusser de Barenne, Garol, and McCulloch,[3] areas 4 and 6 project to the putamen in *Macaca*. This leads to the suggestion that the organization of motor function, which is a prominent feature in primate development may be correlated with a change in the caudate-putamen complex, as well as with the known striking changes in the motor cortex.

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[3] Dusser de Barenne, J. G., Garol, H. W., and McCulloch, W. S., *Research Pub. Assn. Res. in Nerv. and Ment. Dis.*, 1942, **21**, 246.

14411

A Method for Collection of Bacteria from Air and Textiles.*

HENRY M. LEMON. (Introduced by O. H. Robertson.)
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With increasing knowledge of the relationship of infected air and textiles to the dissemination of air-borne diseases, numerous devices for the collection of bacteria have been developed. Some of these are difficult to make (Wells air centrifuge,[1] slit sampler[2]) and others do not appear practical for large scale field studies (textile sampler described by Thomas and Van den Ende[3]). The Moulton atomizer sampler[4] is of relatively simple construction and is easily operated but requires expert glass blowing and is fragile. Furthermore, it has been found unsatisfactory for textile sampling since the lint which collects in the atomizer portion is removed with difficulty.

Application of the Folin aeration tube (as used in the Van Slyke urease method) to the determination of glycol vapor in air by Wise, Puck, and Stral[5] suggested a trial of this inexpensive unit for recovery of air-borne bacteria. Rettger made use of a similar bubbling device[6] which Weinzirl and Thomas

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* This investigation was aided in part through the Commission on Cross Infections in Hospitals, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Division, Office of the Surgeon General, U. S. Army.

[1] Wells, W. F., *Am. J. Public Health*, 1933, **23**, 58.

[2] Bourdillon, R. B., Lidwell, O. M., Thomas, J. C., *J. Hygiene*, 1941, **41**, 197.

[3] Thomas, J. C., and Van den Ende, M., *Brit. Med. J.*, 1941, **1**, 953.

[4] Moulton, S., Puck, T. T., and Lemon, H. M., *Science*, 1943, **97**, 51.

[5] Wise, H., Puck, T. T., and Stral, H. M., *J. Biol. Chem.*, 1943, **150**, 61.

[6] Rettger, L. F., *J. Med. Research*, 1910, **22**, 461.

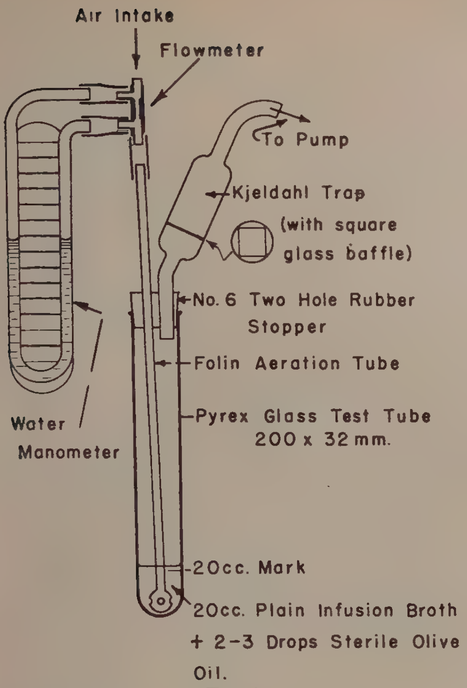
Air Intake
Flowmeter
To Pump
Kjeldahl Trap
(with square glass baffle)
No. 6 Two Hole Rubber Stopper
Folin Aeration Tube
Pyrex Glass Test Tube 200 x 32 mm.
Water Manometer
20cc. Mark
20cc. Plain Infusion Broth + 2-3 Drops Sterile Olive Oil.

FIG. 1.

noted was highly efficient for this purpose.[7]

Description of Apparatus. The apparatus pictured in the accompanying drawing (Fig. 1) consists of a Folin aeration tube[†] made of glass (300 mm long, 6 mm diameter) with a bulb at one end (11 mm diameter) perforated by 6 holes (1 mm diameter) 60° apart in a plane at right angles to its main axis. The tube is passed through a 2-hole No. 6 rubber stopper and the bulb centered near the bottom of the containing test tube (200 mm long, 32 mm in diameter). The Kjeldahl trap with square glass baffle[†] is shortened at both ends for convenience and a slight bend made in the intake, so that this may be inserted into the remaining hole of the stopper. The test tube is scratched at a mark corresponding to 20 cc volume and the Folin tube may be likewise calibrated as a pipette to deliver aliquots from 0.10 to 2.0 cc. A small flowmeter measures the rate of air flow entering the upper open end of the Folin tube.[8] An air pump is attached to the exhaust end of the Kjeldahl trap.[‡]

For sampling of textiles a glass funnel 75-100 mm in diameter is connected to the open end of the Folin tube by means of a piece of rubber tubing with smooth inner wall 300-400 mm long. If certain textiles give off sufficient lint to plug the Folin tube a plain glass tube of identical size may be substituted for it with little loss of efficiency.[§] The apparatus complete with funnel should be mounted in a portable frame by means of clamp holders attached to a piece of metal rod.

Operation. The entire bubbler can be repeatedly autoclaved with the stopper securely in place. The funnel, tubing, flowmeter, and if necessary the bubbler itself can be sufficiently disinfected by rinsing with 70% ethanol and drying. Air drawn in at a rate of 25-30 liters per minute is dispersed through 20 cc of broth containing 2-3 drops of olive oil which inhibits foaming. Room air samples generally require 300 liters of air or more, but 30-60 liters is sufficient for textiles. In sampling the latter, the funnel is slid over the area to be examined, its edges rubbing gently against the fabric, while air is drawn through the material.

Afterwards, the interior of the Folin tube is rinsed by sucking broth up into it. The fluid volume is restored to 20 cc with additional sterile broth. After mixing, suitable aliquots are plated out for bacterial counts.

Collection of Air-Borne Bacteria. This apparatus was compared to the Moulton atomizer sampler (which recovers over 95% of air-borne bacteria) by taking simultaneous air samples at the same location. In addition, the number of microörganisms escaping through the Folin bubbler was determined by passing the exhaust air into a Moulton sampler or a second Folin bubbler (Table I). The Folin bubbler yielded bacterial counts often approximating those obtained from simultaneous tests with the Moulton sampler. These counts represented about 90% recovery

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[7] Weinzirl, J., Thomas, J. B., *Am. J. Pub. Health*, 1913, **3**, 171.

[†] Purchasable at any scientific supply house.

[8] Lemon, H. M., and Wise, H., to be published.

[‡] Suitable pumps obtainable at W. H. Welch Mfg. Co., Chicago.

[§] Suggested by Margaret A. Johnson.

TABLE I.

Collection of Air-Borne Bacteria by Folin Aeration Tube Bubbler.

		Bacterial content of air as simultaneously measured by:		Bacterial content of air leaving Folin bubbler exhaust		
Type of air sampled	Test No.	Moulton atomizer sampler Total bact/ft^3	Folin aeration tube bubbler Total bact/ft^3	Type of sampler used	Total bact/ft^3	% recovery of air-borne bacteria by Folin bubbler
Air of inhabited rooms	1	—	678	Folin	15	98
	2	—	1950	”	26	99
	3	—	169	”	0	100
	4	—	243	”	17	93
	5	—	1480	”	7	99
	6	—	10100	”	34	99
	7	—	2370	”	88	97
	8	—	2230	Moulton	21	99
	9	—	682	”	3	99
	10	896	2290	”	30	99
	11	473	736	”	112	87
	12	122	68	”	11	86
	13	46	33	”	5	87
	14	69	50	”	9	85
	15	948	67	”	23	74
Mean ± S.E.						93 ± 2
		BHS/ft^3*	BHS/ft^3*		BHS/ft^3*	
Air infected with beta hemolytic streptococcus (Group C) dispersed as fine droplets	1	1040	1060	Folin	430	71
	2	1340	1140	”	88	93
	3	706	588	”	37	94
	4	8000	5700	”	935	90
	5	447	343	”	6	98
	6	858	1200	”	64	95
	7	237	225	Moulton	15	94
	8	—	1085	”	172	86
	9	870	570	”	21	96
	10	1320	1480	—	—	
	11	158	141	—		
Mean ± S.E.						90 ± 3

* Beta hemolytic streptococcus.

TABLE II.

Collection of Bacteria from Bedclothes of Scarlet Fever Patients, using Folin Aeration Tube Bubbler. (60 liters of air drawn through textile for each sample.) BHS = beta hemolytic streptococci.

						No. of bacteria recovered	
Patient	Day of hospital stay	Throat culture	Location and type of bedclothes sampled	Bedding, days on bed	Area sampled in ft^2	Total × 1000	BHS*
A.L.	5	BHS	Top sheet, under surface	2	8	—	1,500
			Bottom sheet and pillow	”	”	—	48,000
P.W.	2	BHS	Pillow, both sides	½	8	98	120
			Drawsheet	”	”	1,760	960
			Wool blanket	2	18	710	29,000
T.K.	1	BHS	Pillow, top side	1	2	81	107
			Drawsheet	½	4	204	187
M.S.	10	Neg	Two pillows, both sides	2	12	38	0
			Top sheet, under surface	”	8	82	0
			Drawsheet	”	16	950	0
			Wool blanket	10	12	1,400	80

Note: Drawsheet is placed over central portion of bottom sheet.
* Isolated in 1:500,000 gentian violet rabbit blood agar.

of bacteria suspended in normal room air or air infected with a fine spray of beta hemolytic streptococcus Group C.

Sampling of Bacteria in Textiles. Large numbers of beta hemolytic streptococci and other organisms were collected from bedclothes of scarlet fever patients (Table II), when the device was arranged for textile sampling. Although as convalescence progressed it became increasingly difficult to recover streptococci from blankets and sheets, large numbers of miscellaneous bacteria continued to be obtained. Only a rather small fraction of the total bacterial population is removed by one brief sampling; a second test of the same area gives nearly as high a bacterial count. The number of bacteria recovered was not closely related to the area of sampling, but depended more on other factors now under investigation.

Discussion. Bubbling air through liquids has not been widely used as a means of trapping air-borne bacteria. Some of the previously described methods have been unsuitable for use because of their slow rate of air flow (Rettger's "aeroscope" samples only one-half liter of air per minute), others because of incomplete removal of bacteria which escape to the surface in large air bubbles. The Wheeler bubbler[9] avoids the latter objection by the use of glass beads to break up bubbles, but suitable beads are difficult to obtain and tend to devitrify after prolonged use, with consequent bactericidal alkalinization of the collecting broth. The surprisingly high efficiency of the Folin bubbler in recovering air-borne bacteria may be due to the use of a very rapid air flow, which causes many bacterial particles to impinge on the test tube wall, and produces so great an agitation of the fluid that few large air bubbles are able to rise unbroken to the top of the tube.

Summary. A method is described for collection of air-borne bacteria, by means of dispersing air at a rate of 30 liters per minute from a Folin aeration tube into 20 cc of broth contained in a test tube. The apparatus is about 90% efficient, is readily constructed from simple equipment, and may be adapted to estimation of the bacterial content of textiles.

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[9] Wheeler, S. M., Foley, G. E., and Jones, T. D., *Science*, 1941, **94**, 445.

14412

Essential Fatty Acid Deficiency in the Mouse.

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It was first shown by Burr and Burr[1] that on a diet devoid of fat but otherwise adequate, rats develop deficiency symptoms characterized by retardation of growth, a scaly dermatitis, necrosis of the tail, and kidney lesions and hematuria. Subsequent experiments of the same workers showed that this condition could be cured or prevented by the inclusion in the diet of certain unsaturated fats, such as lard or corn oil, or unsaturated fatty acids such as linoleic acid. These observations have been confirmed by other workers. In the experiments herein described the fat deficiency syndrome has been produced in mice.

Seven weanling rats of the Wistar strain reared in our laboratory were used to check the purity of the diet and to serve as a criterion for the results to be obtained with mice. Their diet consisted of 24% casein, 72% sucrose, and 4% salts (Osborne and Mendel), with daily supplements of 0.65 g of Northwestern Yeast and an equivalent of 70 mg of cod liver oil as the unsaponifiable fraction. After their weight reached 100 g, the protein was reduced to 16% and the sucrose increased proportionately. All dietary constituents and supplements were prepared and rendered fat-free according to the methods of Burr and Burr. On this ration, the rats survived an average of 21 weeks. Slight symptoms of a scaly dermatitis were evident about the seventh week. The ringed, scaly, and necrotic tail condition, and the presence of blood in the urine were not fully apparent until the 17th week. The animals gained about 100 g during the first 10 weeks, after which the rate of growth decreased.

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* Present address: National Oil Products Co., Harrison, N.J.

[1] Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, **82**, 345; 1930, **86**, 587.

The mice used were weanling albino animals from a strain reared in our laboratory over the last 8 years. They were given a diet composed of casein 28%, sucrose 65%, and salts (Osborne and Mendel) 7%, supplemented daily with 0.2 g of Northwestern Yeast and an equivalent of 40 mg of cod liver oil as the unsaponifiable fraction. All material was prepared in the same manner as for the rats. The majority of the forty-seven mice used survived an average of 11 weeks, the others dying during the first few weeks from self-imposed starvation. The animals gained in weight during the first 5 weeks, after which their weight remained fairly constant (Fig. 1). The earliest symptom of the deficiency (30-40th day) was the appearance of a dandruff-like dermatitis at the scruff of the neck, which spread until the fur was filled with the flakes. This scaliness extended to the skin of the ears and extremities. The paws and the base of the tail became red and swollen. As the dermatitis became more severe, the tissues became necrotic, and the animals lost their tail or legs. In a few cases hematuria was observed.

A control group of 16 mice, given the supplemented fat-free diet, into which 10% of lard was incorporated, grew at a faster rate than their experimental litter mates and were still completely healthy and normal in appearance after 120 days on the diet (Fig. 1).

A third group of 4 animals, kept on the fat-free diet until growth had ceased and the dandruff-like dermatitis had developed, were given the lard-containing diet. Growth was promptly resumed and all the symptoms were completely cured (Fig. 2).

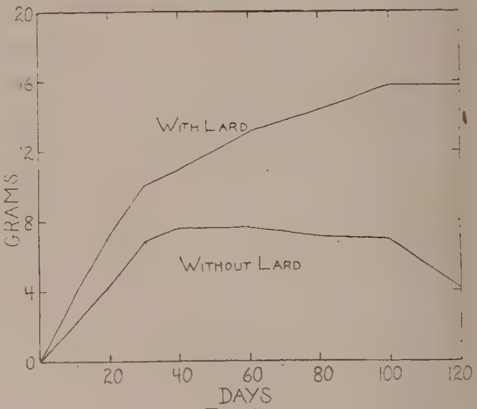

FIG. 1.

Average gains in weight of mice placed at weaning on a diet which was fat-free, and one which contained an added 10% of lard.

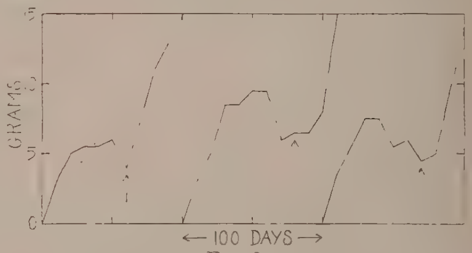

FIG. 2.

Growth response of fat-deficient mice to the addition of 10% of lard to the diet, beginning at the points indicated by the arrows.

Summary. Albino mice kept on a diet devoid of fat developed the Burr and Burr syndrome. The deficiency could be cured or prevented by the administration of lard.

14413

Structural Characteristics of Antifibromatogenic Steroids: Experiments with Acetoxy-Pregnenolone.*

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Abdominal fibroids elicited by estrogens in the uterus[1] and in other parts of the abdominal cavity[2,3] have been shown to be prevented by 4 different steroids present in the body (testosterone, progesterone,[4] desoxycorticosterone,[5] dehydrocorticosterone[6,7]). Attention has been called to the fact that all these substances which possess antifibromatogenic activity are 3-keto-steroids.[8,9,10] Likewise dihydrotestosterone, a 3-keto-steroid not present in the body, was antifibromatogenic.[11,12] On the contrary, androsterone[13] and androstane-3-cis-17-trans-diol[12], steroids with an hydroxyl group at C_3, were not antifibromatogenic.

Since the side chain of two carbons at C_{17} apparently enhances the antifibromatogenic activity of a 3-keto-steroid,[8,9,10] the question arose whether a steroid with an hydroxyl group at C_3 might acquire antifibromatogenic faculty when a side chain of two carbons was added at C_{17}, Δ5-Pregnene-3,21-diol-20-one-21-monoacetate (21-acetoxypregnenolone)† offered the opportunity to study this question.

Tablets of estradiol and of acetoxypregnenolone (abbreviated A.O.P.) were implanted subcutaneously into 15 castrated female guinea pigs. One-half to 4 tablets of A.O.P. were used so as to vary the quantity absorbed. The tablets remained in the body for 90 days. The quantity absorbed was calculated from the loss of weight of the tablets washed and dried in vacuum. Results are given in Table I. The fibrous tumoral reaction was classified according to the system explained in former papers.

The average fibrous tumoral effect (F.T.E.) of A.O.P. acting simultaneously with estradiol was the same as with estradiol alone. The number of animals with abdominal fibroids of a diameter of about 3 mm or more, was also the same with A.O.P. as with estradiol alone. A.O.P. did not prevent other estrogenic or toxic actions of estradiol. The vagina was maintained open throughout the whole course of the experiment. There was genital bleeding and marked growth of the uterus. On the other hand these effects of estradiol are prevented by progesterone or desoxycorticosterone though not always with such small quantities of progesterone as in the present experiment.

The fibrous tumor reaction was still greatly pronounced when more than 300 μg of A.O.P.

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* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, and the Rockefeller Foundation.

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† Thanks are due to Dr. K. Miescher for acetoxypregnenolone.

TABLE I.

Group	Estradiol absorbed per day, μg	Progesterone or A.O.P. absorbed per day μg	No. of animals	No. of animals with tumors classes 2 and 3	Avg fibrous tumoral effect F.T.E.†	Uterine wt, g
Estradiol alone*	16-46	0	15	12	5.1 (2-10.0)‡	5.7 (3.0-12.0)
Estradiol and progesterone*	28-56	Progester. 13-24	14	0	1.4 (1-2.5)	3.0 (1.7-5.1)
Estradiol and A.O.P.	21-63	A.O.P.§ 70-322	15	12	4.8 (1.5-8.0)	4.3 (2.5-8.2)
Estradiol and A.O.P.	26-63	A.O.P. 70-102	5	5	5.8 (2.5-8.0)	5.2 (4.0-8.2)
Estradiol and A.O.P.	30-40	128-202	5	4	4.8 (1.5-7.5)	4.2 (2.5-6.4)
Estradiol and A.O.P.	21-37	248-322	5	3	4.1 (1.5-6.5)	3.6 (2.5-6.2)

* From sets of experiments in [15].

† For an explanation of the units of F.T.E. see Lipschütz *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 164, and other papers.

‡ In parentheses—range.

§ Quantities calculated as free oxypregnenolone.

per day were absorbed. On the contrary, the antifibromatogenic threshold[14] of progesterone is about 13 μg per day;[12] that of desoxycorticosterone acetate is about 90 μg per day.[7,9] The difference between these two steroids and A.O.P. is striking.

A.O.P. is known to have corticoid activity[16,17] only slightly inferior to that of desoxycorticosterone acetate, but its progestational activity is about 25 times less than that of progesterone.[18] Antifibromatogenic activity was apparently concomitant with the progestational one;[9,15] the small progestational activity of A.O.P. would be in accord with the failure of the latter to prevent the fibromatogenic and other toxic actions of estradiol. The fact that A.O.P. has also some estrogenic activity[19] also might be relevant. Because of the great variations one meets in work with experimental fibroids we cannot state whether the diminished F.T.E. and uterine weight in the groups with greater quantities of A.O.P. (see second half of the table) indicate that A.O.P. had a slight antifibromatogenic activity.

Summary. Quantities of acetoxypregnenolone 10 to 20 times greater than the antifibromatogenic threshold of progesterone were unable to prevent the fibromatogenic and other toxic actions of estradiol. This gives new evidence in favor of the following 2 assumptions: first, that the ketonic oxygen at C_3 is essential, or highly important for the antiestrogenic and antifibromatogenic activity of steroid hormones; and second, that the antifibromatogenic activity is often concomitant with the progestational one.

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14414

Cold Agglutinated Erythrocytes: Hemolytic Effect of Shaking.

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The influence of mechanical injury upon the fragility of erythrocytes has not received much attention. The observations reported in this communication indicate an unusual effect of shaking upon cold agglutinated erythrocytes.

In a detailed study Salén[1] has clearly shown that hemolysis of cold agglutinated erythrocytes may occur under certain conditions in the absence of complement. He stressed the fact that the hemolysis differed from the cold-warm hemolysis of Donath and Landsteiner[2] described in syphilitics and in patients with syphilitic cold paroxysmal hemoglobinuria. Other writers (cited in [3]) have commented on the occurrence of hemoglobinemia and hemoglobinuria in cases of cold hemagglutination.

Materials and Methods. Recently observed cases (cited in [3] and [4]) of extremely high titers of cold hemagglutinins have been studied for hemolysis by means of the tests about to be described. In each case hemoglobinemia was readily demonstrated by immersion of an extremity in cold water.

The first was a patient with acute hemolytic anemia without hemoglobinuria during the course of an atypical bronchopneumonia. Six grams of sulfadiazine had been administered 4 days before the apparent onset of the anemia. At the height of the disease the cold hemagglutinin titer was 1/20,000 at 4° C. At this time the *in vitro* hemolytic phenomena, to be described below, were positive. Thirteen days later, when the titer had fallen to 1/800 at 4°C, hemolysis could not be demonstrated.

The second case[4] was a patient with symmetrical gangrene of the tips of the fingers and toes and hemoglobinuria after exposure to moderate environmental cold. The cold hemagglutinin titer of 1/30,000 at 4°C in this instance was constant over many months; the *in vitro* hemolytic tests were always positive.

The experiments were performed within 3 hours of the collection of the blood, except the old and old lyophylized serums mentioned later. Blood or serum was stored at 37°C prior to use. Inactivated serum was heated at 56°C for 1½ hours before use. The old serum* had been collected aseptically 15 months before its use and stored at 6°C. The old lyophylized serum* was also collected 15 months and lyophylized 14 months prior to the experiments. It was made up to its original volume with distilled water before use.

"Blood of the patient" in the experiments refers to the previously mentioned cases with cold hemagglutinins. In all tests homologous or heterologous Group O erythrocytes previously washed with 0.85% sodium chloride solution were employed. The dilutions of the patients' serums were made with 0.85% sodium chloride solution or with normal serum of the same blood group.

All experiments were performed in 10x75 mm serological tubes. The tests were set up and the reactants mixed gently at 37°C. Shaking of the tubes was carried out in a standard manner at room temperature; the tube was held upright at the top between the thumb and index finger of one hand and was tapped at the bottom lightly in rapid succession with the index finger of the other hand. This manipulation was completed in 10 seconds and the tube was replaced in the rack at the temperature of that particular experiment.

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* These serums were collected and prepared in the Littauer Pneumonia Laboratory, Harlem Hospital, New York City, under the direction of Dr. Jesse G. M. Bullowa.

In some of the tests the final readings of hemolysis were made after sedimentation or centrifugation at the temperature of the experiment; in others the results were read after centrifugation at 700 r.p.m. for 2 minutes at room (22°C) temperature.

The term "marked hemolysis" refers to a coloration equivalent to that produced by the complete hemolysis of a 4 to 6% suspension of red blood cells. Since the final erythrocyte concentration was as high as 40% in some experiments (whole blood), hemolysis was never complete.

Results. Summaries of typical experiments which elucidate the mechanism of hemolysis with cold hemagglutination follow:

1. .3 cc of fresh, heat inactivated, old or old lyophylized serum of the patient was mixed with .2 cc 80% suspension of R.B.C. (This was actually a 32% erythrocyte suspension in the final mixture). After incubation at 4°C for 30 min., during which time the tubes were tapped 20 times every 5 min., the tubes were centrifuged. Marked hemolysis was observed.

2. The same experiment as above performed at 4°C without tapping or at 37°C with tapping failed to result in hemolysis. There was marked hemagglutination at 4°C.

3. The same experiment as in (1) carried out with blood serum from normal individuals or from individuals with cold hemagglutinins of titer lower than 1/2500 did not result in hemolysis.

4. The hemolysis of experiment (1) persisted, but in diminishing degree, through a serum dilution of 1/8 and a dilution of the red blood cell suspension of 10% (4% R.B.C. in the final mixture). More dilute serum or red blood cell suspensions did not reveal hemolysis though cold hemagglutination was still marked.

5. .5 cc oxalated whole blood of the patient was kept at 4°C for 30 seconds. At the end of this time the tube was tapped 20 times. No hemolysis was observed after centrifugation.

6. .5 cc oxalated whole blood of the patient was kept at 4°C for 90 seconds. At the end of this time the tube was tapped 20 times. Marked hemolysis was observed.

7. .5 cc oxalated whole blood of the patient was kept at 4°C for 5 minutes. At the end of this time the tube was tapped 2 times. Moderate hemolysis was observed.

8. .5 cc oxalated whole blood of the patient was kept at 4°C for 30 minutes without tapping. The tube was then transferred to the water bath at 37°C. After one minute at this temperature, the tube was tapped 20 times. The tapping was repeated at 5-minute intervals for 30 minutes. After another 30 minutes had elapsed, the tube remaining at 37°C, the tube was centrifuged. No hemolysis was present.

These data demonstrate the existence of a unique type of hemolysis. Concentrated erythrocyte suspensions subjected to marked cold agglutination are readily hemolysed by shaking or tapping. The amount of shaking is not sufficient to hemolyse relatively weakly agglutinated or non-agglutinated erythrocytes. No hemolysis was observed in cases in which the cold hemagglutinin titers were lower than 1/2500 at 4°C. Similarly, light erythrocyte suspensions, even though strongly agglutinated in the cold, were not hemolyzed. Of special interest is the occurrence of hemolysis in the absence of complement. This phenomenon, therefore, is not entirely immunological for lysis requires shaking of the cells but not complement. Hemolysis of cold agglutinated erythrocytes is different from the hemolysis of the Donath-Landsteiner test. In the latter, hemagglutination is slight; erythrocytes sensitized in the cold by amboceptor are hemolyzed at 37°C by complement, lysis failing to occur in the cold.

Summary. These experiments demonstrate an unusual type of hemolysis dependent jointly upon a high concentration of erythrocytes, extreme potency of cold hemagglutination and, presumably, damage as the result of shaking.

14415

Ascorbic Acid in Sweat.

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Some of the values reported in the literature on concentrations of ascorbic acid in sweat indicate that important losses might occur in heavy sweating while other results suggest that losses are negligible. Cornbleet, Klein, and Pace[1] found from 0.55 to 0.64 mg % and Bernstein[2] from 0.5 to 1.1 mg % of ascorbic acid in human sweat. Wright and MacLenathen[3] found 0.024 to 0.186 mg %, while Hardt and Still[4] report 0.18 mg % with higher values up to 0.47 mg % when subjects were given 750 mg of ascorbic acid. Zselyonka and Nánássy-Mégay[5] reported 0.1 to 0.2 mg %. Tennent and Silber[6] found no ascorbic acid but an average hourly loss of 0.23 mg % of dehydroascorbic acid. Mickelsen and Keys[7] report less than 0.1 mg % with an average of about 0.03 %.

In the past, much of the sweat was collected in rubber bags or rubber gloves.[1,7] Mickelsen and Keys[7] obtained results indicating that the type of rubber glove used might make a difference in results. Since the findings of different investigators have been so variable it seemed desirable to run controlled experiments in which the samples were collected in such a manner as to be free from possible interfering substances and also to make comparisons with the sweat collected in rubber bags.

Procedure and Methods. Samples of sweat were collected as follows: In one series of tests the subjects were encased in a rubber bag as far as their necks and seated in a cabinet. Incandescent lamps supplied heat sufficient to induce secretion of 100-150 ml of sweat in 20-30 minutes. In another series of tests the unclothed subjects were seated in the same cabinet, no bag being used. As soon as sweating started the sweat was collected directly into a beaker containing 2-3 g of metaphosphoric acid and thus did not come into contact with anything that could interfere with the ascorbic acid titration. All subjects received 100 mg ascorbic acid daily for 2 weeks prior to tests. To test effects of high vitamin C intake the subjects were given by mouth 500 mg of the vitamin 30-45 minutes before collecting samples.

Ascorbic acid was determined on 10 ml samples according to the method of Bessey and King[8] using 2,6-dichlorophenol indophenol. The oxidase procedure of Tauber and Kleiner[9] for true ascorbic acid was used as a check on interfering substances.

Dehydroascorbic acid was determined on samples of sweat containing 3% HPO_3, which were treated with hydrogen sulphide for one hour, allowed to stand for 30 minutes, the H_2S then swept out with CO_2 or nitrogen and the dye titration carried out. In certain cases dehydroascorbic acid was also determined by the method of Roe and Kuether.[10] The re-

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TABLE I.
Indophenol Titrations of Sweat Collected in Rubber Bag.

Volume of sweat, ml	Ascorbic acid added, mg	Oxidase added, ml	Dye solution used
—	0.5	—	2.20
—	1.0	10	0.01
10	—	—	0.95
10	—	10	0.95
10	0.5	—	3.15
10	0.5	10	0.94

sults checked with those obtained by the dye titration procedure.

Results and Discussion. Table I gives results from tests in which the rubber bag was used in sweat collection. The usual dye titration indicated a rather high ascorbic acid content. However, after treatment with ascorbic acid oxidase, which would destroy any ascorbic acid present, the dye titrations remained the same indicating that the reducing substance present was not ascorbic acid. That this reducing substance was not a constituent of sweat but was derived from the rubber bag was indicated by the fact that the sweat collected directly from the skin in a beaker containing HPO_3 gave no such titration values. As a further check on this, 500 ml of distilled water was placed in the same rubber bag (previously thoroughly washed with soap and rinsed with distilled water) and placed in the same heating cabinet for 30 minutes. Ten ml portions of this water were removed from the bag, 2% HPO_3 added and the dye titration carried out. 0.4 ml of dye were required, equivalent to 0.08 mg ascorbic acid, thus indicating further that the bag was the source of the interfering substances.

TABLE II.
Ascorbic and Dehydroascorbic Acid in Sweat. Subjects on regular diet plus 100 mg ascorbic acid daily. Values expressed in mg per 100 ml.

Subject	Volume of sweat	Ascorbic acid	Dehydro-ascorbic acid
1	150	0	0.05
2	75	0	0.07
3	110	0	0.00
4	100	0	0.08
5	125	0	0.15
6	110	0	0.00
7	105	0.015	0.09
8	95	0	0.06

Table II summarizes the results on the ascorbic acid content of sweat collected directly into metaphosphoric acid solution from subjects on their regular diet supplemented with 100 mg of ascorbic acid per day. It will be noted that while no ascorbic acid was found, small amounts of dehydroascorbic acid appeared to be present. Even if all of the material titrating as dehydroascorbic acid is actually that substance, the amounts present in the sweat were very small.

TABLE III.
Ascorbic and Dehydroascorbic Acids in Sweat. 500 mg ascorbic acid ingested 30-45 minutes before sweating. Values are mg per 100 ml.

Subject	Volume of sweat	Ascorbic acid	Dehydro-ascorbic acid
1	75	0	1.74
2	100	0	1.88
3	95	0	2.13
4	80	0	1.36
5	105	0.054	2.48
6	75	0	1.92

Table III gives some results with subjects ingesting 500 mg ascorbic acid 30-45 minutes before sweating. Some increase in the dehydroascorbic acid concentration was noted but the total elimination was still very low.

Conclusions. Of the samples of human thermal sweat investigated by us only two showed any ascorbic acid present and in these cases the amounts found could be considered practically negligible. Dehydroascorbic acid determinations indicated the presence of this acid in most samples. The amounts found were not sufficient to suggest the induction of vitamin C deficiency through excessive sweating. The use of rubber bags in the collection of sweat may lead to high titration values and appears responsible for some of the high values for ascorbic acid in sweat that have been reported.

14416

Demonstrated Ability of Penile Erection in Castrate Men with Markedly Low Titers of Urinary Androgens.

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Sexual abilities have been described in castrate men (Rowe and Lawrence,[1] Lange,[2] McCullagh and Renshaw,[3] Kolle[4]), but the possibility of some degree of stimulation by androgens from extragonadal sources has remained largely an undetermined factor. After removal of both testes men continue to excrete appreciable quantities of urinary androgens (Gallagher and associates,[5] Callow and co-workers,[6] Hamilton and colleagues;[7] see summary of literature by last authors), and in a rare case the amount excreted is within the range observed in normal men (Callow and co-workers[6]). There is evidence that an androgenic substance found in the urine of normal and castrate men, dehydroisandrosterone, may be derived from secretions of the adrenal glands (Callow,[8] Fraser and associates[9]).

Feinier and Rothman[10] attempted to define the quantity of urinary androgens in a 53-year-old castrate who claimed erectile ability, but the description of their findings seems confusing. They reported an entire absence of androgens in the urine, although, as indicated above, some quantity of these substances is found almost invariably with the use of adequate methods of extraction and assay. Description of their technic was omitted. They commented that neither testosterone nor androsterone was present in extracts obtained apparently from 3,000 cc of urine, an odd statement since testosterone has never been found in urine. It would have been difficult to identify specific compounds present in this small quantity of urine, and since the extract appears to have been used for assay in capons, it is doubtful that the authors intended to imply that the isolation of compounds was attempted.

The present communication pertains to 2 men castrated for 13 and 18 years, respectively, who demonstrated during repeated examinations a pronounced degree of erectile ability despite the absence of any considerable stimulation by androgens from extragonadal sources, as indicated by low titers of urinary androgens and supranormal values for urinary gonadotropins.

This marked capacity for penile erection distinguished these men from 6 other members of a group of 8 eunuchs all of whom have been studied during periods of one to 5 years with repeated physical examinations and bioassay of urinary androgens, estrogens, and gonadotropins. In view of the difficulties encountered in evaluating the sexual status in castrate men, especially with regard to the reliability of the history obtained from the patient and the interplay of psychogenic and endocrine factors, daily records have been kept by each person, tabulating the number, duration and completeness of erections, and the performance of intercourse. These records were collected weekly and where possible were compared with statements obtained from their partners in intercourse.

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The methods and results of bioassays of urinary androgens, estrogens, and gonadotropins, which were made in collaboration with Drs. R. I. Dorfman, H. R. Catchpole, and G. R. Hubert, are described in detail elsewhere (Hamilton, Dorfman, and Hubert;[7] Catchpole, Hamilton, and Hubert[11]). During intervals in which urine was collected the patients were ambulatory and in good health. Determinations of androgenic and estrogenic substances were made upon pooled samples of urine each of which represented collections made for 4 consecutive 24-hour periods and preserved with toluene. Within 2 weeks after collection the specimens were extracted with benzene in a continuous extractor according to the method of Gallagher and associates.[5] The growth of combs in day-old chicks (Dorfman and Greulich[12]) was used in assays of androgenic activity, which are expressed in international units (I.U.), the unitage of activity being that evoked by 0.1 mg of androsterone. A minimum of 15 and usually 20 animals was used for each assay. Determinations are held to be accurate within ± 25%.

The alkali soluble fraction of the urinary extract was assayed for estrogenic activity in adult spayed female mice. The results are expressed in international units (I.U.), one unit being equal to the reaction elicited by 0.1 μg of estrone. The accuracy of the method is considered to be ± 50%.

Values for gonadotropic substances were determined separately in samples of urine each collected over a period of 24 hours. One cc of toluene was added to each specimen which was kept chilled until extracted within 24 to 48 hours by the method described by Freed and Hechter.[13] Bioassay was performed separately on each daily sample, utilizing changes in the uterine weight of 21- to 23-day-old mice in accordance with Catchpole's modification[14] of the technic described by Levin and Tyndale.[15] The values are defined in terms of a mouse unit (M.U.), which is that amount of substance that under standard conditions doubles the ratio of uterine weight/body weight in 21- to 23-day-old female mice of our stock.

Abstracts from the case histories are as follows:

Case one was a 43-year-old man who, when 25 years of age, had been castrated surgically because of suspected cancer. He stated that penile erections had occurred in the years prior to castration and had continued to appear throughout the years subsequent to orchidectomy.

The patient was a man of medium build with pale, finely wrinkled skin and a tendency to obesity. Deposits of fat were evident over the mammæ, trochanters, pubis, and lower abdomen. Muscular prominences and ridges were not obvious, and the contour of the face, trunk, and limbs was smooth. Hair over the body was scant, consisting of a few silky strands in the axilla, and isolated hairs of the terminal type on the chin and on the inner side of the lower and upper leg. Terminal hairs about an inch in length were distributed sparsely over the pubis.

The external genitalia had not regressed to an eunuchoid state, but the scrotum and the glans and shaft of the penis were poorly distinguished by ruddiness or pigmentation from skin of the thigh. The penis measured 9 cm in length when flaccid. The scrotum had only slight rugosity and when cooled did not become deeply furrowed. Upon rectal palpation the prostate was felt to be small and nonedematous. No secretion was obtained upon massage of the prostate. Urethroscopy and urological examination revealed no condition that seemed to be related to erectile ability of the penis.

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TABLE I.
The titers of urinary hormones of the men described in this article are typically eunuch-like in that the values for androgens and estrogens are subnormal and those for urinary gonadotropins are supranormal.

		Androgens		Estrogens		Gonadotropins	
Subjects (Type and No.)	Citation	No. days tested	Avg (I.U.)	No. days tested	Avg (I.U.)	No. days tested	Avg (M.U.)
Case 1		5	8	4	28	10	130
Case 2		4	11	4	15	10	40
Eunuchs (2)	16	18+	1.66 and 5.8 (per liter)	18+	30 and 45 (per liter)		
(2)	7, 11	4	9 and 3	4	11 and 5	10	50 and 60
Normal men (4)	5	39-45	63-68	39-45	90-120		
(5)	17					30-120	8, 11, 12, 15 and 25

When the patient stood undressed before examination was begun, erection of the penis occurred until the organ attained an angle of about 80°. The penis did not appear to be flushed during erection and the glans was of a light gray color. The shaft of the penis was firm throughout its length and resistant to strong downward pressure. The erection persisted for slightly more than a minute, then slowly subsided.

During examination at later periods complete erections were observed on several occasions. The patient stated that this phenomenon occurred quite commonly and in some circumstances when he was unaware of external stimulation.

The average daily titers of urinary androgens and estrogens were lower than in normal men, whereas those of urinary gonadotropins were supranormal (Table I). On 2 of the days in which urine was collected for extraction and assay of hormones, erections were observed. The results obtained with routine urinary and blood analyses were not remarkable. On 3 occasions the BMR was –5, –10, and 7.

Case two was a 39-year-old man whose testes and scrotum had been removed surgically after accidental traumatic injury at the age of 26 years. Other than phenomena known to accompany castration (see Hamilton[18]), some of which have been described in Case 1, there was little of special interest revealed upon clinical and laboratory examination. The penis was 10 cm in length in a flaccid state. No fluid was obtained with prostatic massage.

Complete penile erection occurred at the first examination upon inspection of a small inguinal hernia and during 2 of a subsequent series of examinations. The erections were maintained for periods of a minute or longer, during which time the skin of the penis remained rather light in color and the glans was a pale gray.

The history related by the patient was in agreement with that obtained separately from his wife to the effect that intercourse had been carried out 2 or 3 times weekly previous to castration and during all 13 years since castration. Intercourse during the next 6 months as tabulated from records kept daily was carried out one to 4 times per week. The sexual interest shown by this patient appeared to be considerable according to the history obtained from the man, his wife, and a third party concerned. The patient claimed that orgasms occurred but that the ejaculate consisted of only about a drop of fluid.

The titers of urinary hormones were in the range typical of castrates (Table I).

In these eunuchs the low values for urinary androgens and estrogens and the supranormal quantities of urinary gonadotropins are evidence that the state of testicular insufficiency

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[16] Kenyon, A., Gallagher, T., Peterson, D., Dorfman, R., and Koch, F., *J. Clin. Invest.*, 1937, **16**, 705.

[17] Werner, S. C., *J. Clin. Invest.*, 1943, **22**, 395.

[18] Hamilton, J. B., *J. A. M. A.*, 1941, **116**, 1903.

has not been compensated for in a material fashion by androgens derived from extragonadal sources. That the titers of these urinary hormones would reflect such androgenic stimulation as was present, was indicated when at a later date these men received daily intramuscular injections of 20 mg of testosterone propionate in one cc of peanut oil. With this treatment, which appears to provide only a minimal degree of replacement therapy (Hamilton;[18] Catchpole, Hamilton, and Hubert[11]), the quantities of urinary androgens and estrogens were increased to lie within the range found in normal men (Hamilton, Dorfman, and Hubert[7]) and the amounts of urinary gonadotropins were reduced to normal or subnormal limits (Catchpole, Hamilton, and Hubert[11]).

Comment. Two men who had been completely castrate for 13 and 18 years, respectively, had a marked capacity for penile erection, as observed during physical examinations and as tabulated in records kept daily for a year. Statements as to the interest and ability in intercourse exhibited by one of these men who was married were corroborated by his wife. Low titers of urinary androgens and clinical and laboratory evidence of testicular insufficiency, including supranormal values for urinary gonadotropins, provide assurance that there was no considerable androgenic secretion derived from extragonadal sources.

It would appear, then, that a marked capacity for penile erection may continue for many years subsequent to castration and that this phenomenon is not the result of stimulation by androgens derived from extragonadal sources because it may occur in individuals with definitely subnormal titers of androgens in the bodily tissues and fluids. It is not suggested that this phenomenon is typical of the majority of castrate men.

14417

Anesthesia: XI. The Anesthetic Action of Propenyl Ethyl Ether.*

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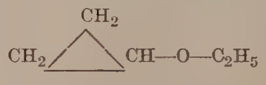
In former studies[1] the authors showed that cyclopropyl ethyl ether (cypreth ether) was an anesthetic agent in many species of animals. Attempts to induce surgical anesthesia in man with this agent were not satisfactory.[2] In man, the concentration of the vapors of cypreth ether required to induce anesthesia were irrespirable. It occurred to us to prepare an isomer of this true hybrid molecule between cyclopropane and ethyl ether.

$$CH_3 \cdot CH : CH \cdot O \cdot C_2H_5$$

Propenyl Ethyl Ether



Cypreth Ether

Propenyl ethyl ether is a volatile, colorless liquid, with a characteristic ethereal odor; it boils at 69°C and has a specific gravity of 0.774 at 20°C. At 25°C, 0.5 cc of this ether dissolves in 100 cc of water and the compound is not hydrolyzed at pH 7.4 in a phosphate buffer. Concentrations of the vapors of propenyl ethyl ether sufficient to produce

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* The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Co. of Cleveland, Ohio.

[1] Krantz, J. C., Jr., Carr, C. J., Forman, S. E., Evans, W. E., Jr., and Wollenweber, H. L., *J. Pharm. and Exp. Therap.*, 1941, **72**, 233.

[2] Kilborn, M. G., Forman, S. E., Evans, W. E., Jr., and Krantz, J. C., Jr., *Anesthesiology*, 1942, **3**, 414.

incipient narcosis were found to be irritating to man.

Observation Anesthesias (monkey). Two large Rhesus monkeys were each anesthetized twice with propenyl ethyl ether. The induction period was short and uneventful. During induction and also during the period of surgical anesthesia much mucus accumulated in the bronchial tree. Relaxation of the musculature of the abdomen and extremities was complete under surgical anesthesia. The recovery period was prompt.

Anesthetic Index (dog). The general plan of administering the anesthetic was the same as that described previously.[3] The number of cubic centimeters per kilogram required to produce surgical anesthesia was divided into the volume per kilogram required to produce respiratory arrest and the quotient designated as the anesthetic index. In 10 dogs, the mean value per kg required to induce anesthesia was 0.45 cc while 1.43 cc per kg produced respiratory failure. The mean anesthetic index was determined to be 3.18. Two animals failed to recover when artificial respiration was instituted. There was sneezing, coughing, and much mucus produced in most of the dogs.

Blood Pressure Studies (dog). In two animals the blood pressure fell to about three-quarters of its preanesthetic value and remained at that level until approaching respiratory failure. After respiratory arrest the heart continued to beat several minutes. Under anesthesia with propenyl ethyl ether 4 dogs were cardioscoped and electrocardiograms made in the light and deep planes of surgical anesthesia. In this series of dogs, not any animal revealed any significant differences in rate, form, or regularity of the E.C.G. The dilution of the compound in Howell-Ringer's solution which produced the liminal effect on the frog's heart within one minute was determined to be approximately 0.02 molar.

Concentration Required for Anesthesia (mouse). The concentration of propenyl ethyl ether required to induce anesthesia was determined by typical partial pressure experiments described previously.[3] In 48 mice, 50% of the animals were anesthetized at a partial pressure of 2.5%, while 3% partial pressure produced anesthesia in all animals. With ethyl ether, in former studies 4% partial pressure did not produce anesthesia and 5% anesthetized 40% of the animals.

Delayed Anesthetic Deaths (rat). Twelve male adult rats were anesthetized with propenyl ethyl ether to the surgical plane and maintained in this condition for one-half hour. Two animals were sacrificed immediately thereafter for histologic study. The remaining 10 animals lived and were in good nutritional state over a period of 28 days observation.

Histologic Studies of Viscera (dog and rat). Two of the rats used in the delayed death studies were used for histologic studies of the kidneys and liver. There were no abnormalities attributable to the anesthetic. Three dogs were anesthetized lightly with cyclopropane and liver biopsies performed. The anesthetic agent was changed to propenyl ethyl ether and anesthesia maintained for from one to 2 hours and a second hepatic biopsy performed. There were no significant abnormalities.

Hemolysis Studies (dog). Ten cc volumes of propenyl ethyl ether of varying concentrations in normal salt solution were maintained at 25°C. To each of these was added 0.1 cc of defibrinated dog's blood and the time for hemolysis observed. Concentrations of 22, 43, and 84 mg % produced no hemolysis within 12 hours. A saturated solution of the agent in normal salt solution produced hemolysis within 5 hours at 25°C.

Summary. Propenyl ethyl ether produces prompt surgical anesthesia in many species of animals upon inhalation. The anesthetic index is higher than ethyl ether and the compound appears to be without deleterious action upon the myocardium, the normal histology of the liver or kidney, or upon the red blood cell in anesthetic concentrations. However, this ether in common with its isomer, cypreth ether, produces irritation of the upper respiratory tract in animals and especially in man. For this reason we are of the opinion that the compound is unsuited as a volatile anesthetic in man.

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[3] Krantz, J. C., Jr., Carr, C. J., Forman, S. E., and Evans, W. E., Jr., *J. Pharm. and Exp. Therap.*, 1940, **69**, 207.

[4] Krantz, J. C., Jr., Carr, C. J., Evans, W. E., Jr., and Forman, S. E., *J. Pharm. and Exp. Therap.*, 1943, **78**, 115.

14418

The Serum Potassium in Two Cases of Sprue.

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Studies of sprue and allied conditions in recent years have shown that many elements of the diet are poorly absorbed or poorly utilized. The dietary constituents chiefly emphasized have been fat, carbohydrate, protein, calcium, phosphorus, and vitamins A, D, K, and parts of the B-complex. In some instances the defective absorption of a particular element of the diet is reflected in serum values lower than normal. This is notably true for calcium. To our knowledge no previous investigation of the values of serum potassium in sprue-like syndromes has been carried out. We have recently observed 2 cases of sprue in which the serum potassium was found to be at extraordinarily pathological levels. The blood chemical findings in these 2 patients at the time of admission to the hospital are the subject of this report. Determinations of the serum potassium were carried out by the method described by Harrison and Darrow.[1]

Case 1 was a 53-year-old Spaniard who complained of diarrhea, a sore tongue and extreme weakness for 3 weeks prior to his hospital admission. He was markedly emaciated, weighing only 37 kg (81.4 lbs), and gave the appearance of having been ill much longer than his history would indicate. His tongue was smooth and reddened. He was having 5 or 6 loose bowel movements a day, and the stools were found to contain excessive amounts of fat. The oral glucose tolerance test showed a low curve. He had achlorhydria and a severe, macrocytic, hyperchromic anemia. The blood chemical values are shown in Table I. It will be noted that the serum potassium level was 1.1 meq/L (4.3 mg/100 cc), as compared with the normal range of approximately 4.1 meq/L (16 mg/100 cc) to 5.6 meq/L (22 mg/100 cc).

Case *2* was a 44-year-old Greek woman, whose symptoms of sprue were later attributed to lymphosarcoma of the intestine and mesenteric lymph nodes. She had, during this time, 3 to 8 bulky, frothy, foul-smelling stools daily. She had had a moderate anemia which had responded somewhat to treatment with liver and transfusions, without general improvement. During her entire illness she had complained of weakness, which on admission was so severe that she could not feed herself or sit up unaided. The patient was emaciated, weighing 40 kg (88 lbs), and was extremely weak, although no true paralysis was present. She had a marked steatorrhea. The oral glucose tolerance test showed a low curve. The Chvostek and Trousseau signs were positive. The blood chemical values are shown in Table I. On admission the serum potassium was 1.3 meq/L (5.1 mg/100 cc) and 5 days later was even lower, 1.1 meq/L (4.3 mg/100 cc).

These values for the serum potassium, 1.1 and 1.3 meq/L are almost, if not quite unique. Abnormally low levels have been reported previously in several different clinical conditions. First, and most commonly, low serum potassium has been encountered in familial periodic paralysis. It was first reported by Biemond and Daniels[2] who found a level of

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[1] Harrison, H. E., and Darrow, D. C., *J. Biol. Chem.*, 1937, **121**, 631.

[2] Biemond, A., and Daniels, A. P., *Brain*, 1934, **57**, 91.

TABLE I.
Blood Chemical Values in Two Cases of Sprue.

	Date	Serum K meq/L	Serum K mg/100 cc	Serum Ca mg/100 cc	Serum P mg/100 cc	Serum Na meq/L	Serum Protein g/100 cc
Case 1	4/1/41	1.1	4.3			138.0	5.2
	4/3	1.6	6.2	6.1	1.1	139.0	
Case 2	7/23	1.3	5.1	4.8	2.2	139.8	5.0
	7/28	1.1	4.3		1.9		

13.38 mg % (3.4 meq/ L) in one patient during an attack of paralysis. The association has been repeatedly confirmed by many observers. So far as we know the lowest level of serum potassium previously recorded in the disease was noted by Allott and McArdle[3] who gave epinephrine subcutaneously to a patient with familial periodic paralysis and precipitated an attack. Coincident with the paralysis there was a fall in the serum potassium level to slightly over 5 mg/100 cc (1.3 meq/L). The same authors also noted a marked diminution in serum potassium in normal individuals after epinephrine was given in repeated doses. Harrop and Benedict[4] reported low levels of the serum potassium in diabetics who were receiving insulin therapy, the lowest level being 8.5 mg/100 cc (2.2 meq/L). Ferrebee, Ragan, Atchley, and Loeb[5] reported a case of Addison's disease under treatment with desoxycorticosterone in which the serum potassium fell as low as 2.9 meq/L (11.3 mg/100 cc).

In view of many observations in animals on the physiological effects of changes in the potassium ion concentration in tissues one would expect symptoms of great severity in these conditions where serum potassium levels were so low. Both of our patients were extremely ill at the time these findings were noted. Both exhibited generalized muscular weakness, which in the second patient was so severe that she could not sit up unaided and was unable to raise her arms from the bed. It seems not unlikely that in part this weakness may have been related to the changes in the serum potassium and that it might have been still more profound had not the effects of the low serum potassium been modified by the associated low serum calcium. It is perhaps worthy of note that spasticity of the muscles which might have been expected with low serum calcium values was not present in either case.

Summary. Two cases of sprue are reported in which the values of the serum potassium were found to be at low levels, 1.1 meq/L to 1.6 meq/L, before the institution of treatment.

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[3] Allott, E. N., and McArdle, B., *Clinical Science*, 1937-38, **3**, 229.

[4] Harrop, G. A., Jr., and Benedict, E. M., *J. Biol. Chem.*, 1924, **59**, 683.

[5] Ferrebee, J. W., Ragan, C., Atchley, D. W., and Loeb, R. F., *J. A. M. A.*, 1939, **113**, 1725.

14419

Genetic Theory of the Rh Blood Types.*

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In a previous paper,[1] a classification of human bloods according to their reactions with 2 principal varieties of anti-Rh agglutinins was proposed, in which 3 sorts of Rh-positive blood are distinguished, designated as Rh_1, Rh_2, and Rh′. Furthermore, it was demonstrated that the 3 most common types, Rh_1, Rh_2, and Rh-negative, are inherited by triple allelic genes, Rh_1, Rh_2, and *rh*. Recently, Wiener and Sonn[2] described a new variety of anti-Rh agglutinin reacting with about 35% of bloods from white individuals in New York City. The agglutinogen detected by this isoantibody proved to be inherited as a simple Mendelian dominant by means of a special gene allelic to *rh*. With the new agglutinogen the number of subdivisions within the Rh-positive type has been further increased, and the purpose of the present communication is to present a more complete classification and genetic theory in order to include these additional types.

In Table I is presented once more the classification of the 4 types of blood defined by the standard anti-Rh serum of Landsteiner and Wiener,[3] reacting with about 85% of bloods from white individuals, and the anti-Rh_1 serum of Wiener,[4] giving about 70% positive reactions. This table is similar to that given in the previous paper.[1] As Wiener and Landsteiner[1] have pointed out, type Rh_1 and type Rh_2 bear a serologic and genetic relation to each other similar to that of the subgroups A_1 and A_2. For example, the reactions of type Rh_1 blood with the 2 agglutinins anti-Rh and anti-Rh_1 are not due to 2 genetically distinct antigens Rh and Rh_1 in such blood, but to an agglutinogen Rh_1 containing 2 "partial" antigens inherited as a unit by means of a corresponding gene. The analogy

TABLE I.
Relation Between Agglutinins Anti-Rh and Anti-Rh_1.
(Modified after Wiener and Landsteiner.)

Antiserum	Agglutinins in serum	Types of blood and approximate distribution in N.Y.C. (whites)			
		"Rh_1" 67%	"Rh′" 2%	"Rh_2" 18%	"Neg." 13%
Anti-Rh* (standard)	Anti-Rh	Pos.	Neg.	Pos.	Neg.
Anti-Rh_1†	Anti-Rh_1	Pos.	Pos.	Neg.	Neg.
Anti-Rh′‡	Anti-Rh and Anti-Rh_1	Pos.	Pos.	Pos.	Neg.

Quotation marks are used in order to distinguish the 4 types of this table from the more completely classified types of Table IV. Each type in this table includes 2 types of Table IV. For example, the "Neg." type here also includes type Rh″ of Table IV; type "Rh_1" here includes types Rh_1 and Rh_1Rh_2 of Table IV, etc.

* Landsteiner, K., and Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1940, **42**, 223.

† Wiener, A. S., *Arch. Path.*, 1941, **32**, 227; Landsteiner, K., and Wiener, A. S., *J. Exp. Med.*, 1941, **74**, 309.

‡ Wiener, A. S., *Arch. Path.*, 1941, **32**, 227; Levine, P., Burnham, L., Katzin, E. M., and Vogel, P., *Am. J. Obst. and Gyn.*, 1941, **42**, 925.

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* Aided by grants from the Carnegie Corporation through the Committee on Human Heredity of the National Research Council, and from the United Hospital Fund of New York City.

[1] Wiener, A. S., and Landsteiner, K., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 167.

[2] Wiener, A. S., and Sonn, E. B., *J. Immunol.*, in press; Wiener, A. S., *Science*, 1943, **98**, 182.

[3] Landsteiner, K., and Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1940, **42**, 223.

[4] Wiener, A. S., *Arch. Path.*, 1941, **32**, 227.

TABLE II.
Relation Between Agglutinins Anti-Rh and Anti-Rh_2.

Antiserum	Agglutinins in serum	Types of blood and approximate distribution in N. Y. C. (whites) I 32.5%	II 0.5%	III 52.5%	IV 14.5%
Anti-Rh (standard)	Anti-Rh	Pos.	Neg.	Pos.	Neg.
Anti-Rh_2*	Anti-Rh_2	Pos.	Pos.	Neg.	Neg.
Anti-Rh″†	Anti-Rh and Anti-Rh_2	Pos.	Pos.	Pos.	Neg.

*Wiener, A. S., and Sonn, E. B., *J. Immunol.*, in press; Wiener, A. S., *Science*, 1943, **98**, 182.

† Wiener, A. S., unpublished observations. This type of serum is difficult to distinguish from standard anti-Rh serum unless blood of the rare type Rh″ is available.

to the subgroups of A is not complete, however, because rare bloods exist which react with anti-Rh_1 but not anti-Rh agglutinins, and the so-called agglutinogen Rh′ in these bloods, as will be shown presently, is determined by a special allelic gene. The reason why the anti-Rh′ sera (containing both agglutinins anti-Rh and anti-Rh_1) are the most common among the human Rh antisera is that an Rh-negative mother with an erythroblastotic infant of the most frequent type Rh_1 would be apt to possess agglutinins specific for both of the partial antigens in Rh_1 blood. In many anti-Rh′ sera, the strength of the two agglutinins is unequal, and most of the so-called anti-Rh_1 sera are really anti-Rh′ with very weak anti-Rh agglutinins which do not react under the conditions of the test.

As is shown in Table II, the anti-Rh_2 agglutinin of Wiener and Sonn bears a relation to the standard anti-Rh agglutinin entirely analogous to that of the anti-Rh_1 agglutinin. The agglutinogen in blood of type I, like the Rh_1 agglutinogen, appears to possess 2 "partial" antigens, and these are inherited together as a unit by means of a special allelic gene. The rare type II is analogous to type Rh′; it reacts with anti-Rh_2 but not with anti-Rh agglutinins and presumably is also determined by a special allelic gene. Human sera containing anti-Rh_2 agglutinins, as a rule, also contain anti-Rh agglutinins (anti-Rh″ serum of Table II), for reasons already presented; in fact, anti-Rh_2 sera are really anti-Rh″ sera with weak anti-Rh agglutinins.

Incidentally, human sera containing both the agglutinins anti-Rh_1 and anti-Rh_2 have not yet been encountered and such sera must be rare. The reason for this is that Rh-negative mothers cannot have type Rh_1Rh_2 children, although if such a mother has a type Rh_1Rh_2 husband, half the children will be Rh_1 and half Rh_2. At any rate, isoimmunization against both Rh_1 and Rh_2 cannot occur during the same pregnancy. Simultaneous isoimmunization against Rh_1 and Rh_2 could occur, however, in an Rh-negative individual receiving transfusions of type Rh_1Rh_2 blood.

If the reactions of the 3 agglutinins, anti-Rh, anti-Rh_1, and anti-Rh_2, are considered together, five sorts of Rh agglutinogens can be differentiated, each presumably determined by a corresponding allelic gene (*cf.* Table III). These, together with the recessive gene, make up a series of 6 allelic genes, Rh_1, Rh_2, *Rh*, *Rh′*, *Rh″*, and *rh*. As is shown in Table IV, these six genes give rise to 21 theoretically possible genotypes, corresponding to 8 different types of blood, of which, to date, 7 have already been encountered. The eighth type Rh′Rh″ corresponds to the genotype *Rh′Rh″*, the calculated frequency of which is very low. Therefore, it is not surprising that blood giving the reactions of the theoretical type Rh′Rh″ has not yet been found.

Investigations on family material[5] to test this theory of 6 allelic genes have been carried out, and the results in 92 families with 267 children conform with the theoretical expectations. A few illustrative observations will be cited:

(1) In every case where a child possesses agglutinogen Rh_1 or Rh_2, one or both parents also possess the corresponding agglutinogen. Moreover, no instance has yet been encoun-

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[5] Wiener, A. S., Sonn, E. B., and Belkin, R., *J. Exp. Med.*, in press.

TABLE III.
Variants of the Rh Agglutinogen and Their Corresponding Genes.

Agglutinogen* in erythrocytes	Reactions with Rh Antisera					Designation of genes*
	Anti-Rh	Anti-Rh$_1$	Anti-Rh$_2$	Anti-Rh$'$	Anti-Rh$''$	
Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	rh
Rh$_1$	Pos.	Pos.	Neg.	Pos.	Pos.	Rh_1
Rh$_2$	Pos.	Neg.	Pos.	Pos.	Pos.	Rh_2
Rh	Pos.	Neg.	Neg.	Pos.	Pos.	Rh
Rh$'$	Neg.	Pos.	Neg.	Pos.	Neg.	Rh'
Rh$''$	Neg.	Neg.	Pos.	Neg.	Pos.	Rh''

* See foot-note to Table IV.

TABLE IV.
The Eight Rh Blood Types and Their Theoretical Genotypes.

Rh Type* (Phenotype)	Reactions with Rh antisera					Approximate frequency in N.Y.C. (whites) %	Theoretically possible genotypes*
	Anti-Rh	Anti-Rh$_1$	Anti-Rh$_2$	Anti-Rh$'$	Anti-Rh$''$		
Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	13.0	$rhrh$
Rh$_1$	Pos.	Pos.	Neg.	Pos.	Pos.	50.0	Rh_1Rh_1 Rh_1rh Rh_1Rh Rh_1Rh' $Rh'Rh$
Rh$_2$	Pos.	Neg.	Pos.	Pos.	Pos.	15.5	Rh_2Rh_2 Rh_2rh Rh_2Rh Rh_2Rh'' $Rh''Rh$
Rh$_1$Rh$_2$	Pos.	Pos.	Pos.	Pos.	Pos	17.0	Rh_1Rh_2 Rh_1Rh'' $Rh'Rh_2$
Rh	Pos.	Neg.	Neg.	Pos.	Pos.	2.5	$RhRh$ $Rhrh$
Rh$'$	Neg.	Pos.	Neg.	Pos.	Neg.	1.5	$Rh'Rh'$ $Rh'rh$
Rh$''$	Neg.	Neg.	Pos.	Neg.	Pos.	0.5	$Rh''Rh''$ $Rh''rh$
Rh$'$Rh$''$	Neg.	Pos.	Pos.	Pos.	Pos.	—†	$Rh'Rh''$

* Knowledge concerning the Rh factors is changing so rapidly that the system adopted in the present paper should only be regarded as provisional and temporary.

† This theoretically possible type has not yet been encountered, which is not surprising considering the extremely low value of the calculated frequency of the genotype $Rh'Rh''$.

tered of a type Rh$_1$Rh$_2$ parent with an Rh-negative child or an Rh-negative parent with a type Rh$_1$Rh$_2$ child.

(2) An Rh-negative mother and type Rh$_1$Rh$_2$ father had 5 Rh$_1$ and 5 Rh$_2$ children. According to the theory, this father belongs to genotype Rh_1Rh_2, and as expected, half the children belong to genotype Rh_1rh and half to genotype Rh_2rh.

(3) In 2 families Rh$_1$ × Rh-negative, there was a total of 7 type Rh$_1$ and 5 Rh-negative children. Here the Rh$_1$ parents obviously belong to genotype $Rh_1\ rh$.

(4) In a family Rh$_1$ × Rh$_1$, there were 3 Rh$_1$ and 2 Rh$'$ children. Here, evidently, one of the Rh$_1$ parents belongs to genotype Rh_1Rh', the other to genotype Rh_1Rh' or genotype Rh_1rh.

(5) Families Rh_1 × Rh_1 and Rh_1 × Rh-negative have been encountered in which the children belonged either to type Rh_1 or type Rh. In these families one or both of the Rh_1 parents must belong to genotype Rh_1Rh.

(6) In a Negro family, the husband belonged to type Rh′, the child to type Rh_2 while the wife was Rh-negative, in conflict with the heredity theory. However, the husband was actually not the father of the child because he belonged to type N and the child to type M.

These examples suffice to illustrate how the theory operates and the complete family data will be presented in detail in a separate publication.[5]

An interesting consequence of the theory is that an antigen resulting from the action of a single gene may be indistinguishable serologically from that produced by the combined action of two allelic genes. Thus, gene Rh_1 alone produces the same effect as genes *Rh* and *Rh′* together, and gene Rh_2 produces the same effect as genes *Rh* and *Rh″* together. The only way to differentiate the two cases is by family studies. For example, in the mating Rh-negative × Rh_2, if the Rh_2 type is determined by gene Rh_2, in some families all the children will belong to type Rh_2, while in other families half the children will be Rh_2 and half Rh-negative (or type Rh). On the other hand, in matings Rh-negative × Rh_2 where the Rh_2 parent belongs to genotype *RhRh″*, half the children will belong to type Rh and half to type Rh″.

The Rh blood types should prove useful in forensic medicine for criminal identification because they multiply the number of individual differences in human blood. Moreover, the Rh types offer the prospect of increasing substantially the chances of exclusion in cases of disputed parentage.

In typical cases of erythroblastosis where the mother is Rh-negative and the father is Rh-positive, if the father belongs to type Rh_1Rh_2 the prognosis for future pregnancies is poor because every child must be Rh-positive (either type Rh_1 or type Rh_2). Moreover, should specific desensitization prove feasible as a method of preventing erythroblastosis, it might be found essential to use Rh antigens of the same variety as that to which the mother has been sensitized.

In conclusion, it should be mentioned that the number of Rh types existing is not limited to those described in this paper. For example, occasionally bloods are encountered which give intermediate reactions, and rare bloods have been found which give atypical reactions.

Summary. Three varieties of anti-Rh agglutinins have been encountered, designated as anti-Rh (standard), anti-Rh_1, and anti-Rh_2, and agglutinating, respectively, approximately 85%, 70%, and 35% of bloods from white individuals in New York City. Human sera containing agglutinins anti-Rh and anti-Rh_1 together are designated anti-Rh′; sera containing the two agglutinins anti-Rh and anti-Rh_2 are designated anti-Rh″. By means of the 3 sorts of Rh agglutinins, 5 varieties of Rh agglutinogens are demonstrable, designated as Rh_1, Rh_2, Rh, Rh′, and Rh″. These in combination give rise to 8 Rh blood types (including the Rh-negative type), of which all but one, the rarest, have actually been encountered. A theory to explain the heredity of the Rh blood types is proposed in which the existence of 6 allelic genes is postulated. Evidence in support of the theory is presented and some of the implications of the theory are mentioned.

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Nucleated Erythrocytes in Newly-born Infants in Relation to Maternal Rh Compatibility.

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Studies on the incidence of nucleated erythrocytes in newly-born infants have not taken into account the possible incompatibility between mother and child as to the recently described Rh factor. When this blood factor[1-4] is present in the infant but not present in the mother's blood, the mother may develop antibodies against it, and abortion, jaundice, erythroblastosis fetalis and ill-defined conditions may sometimes develop.[3,5] It seemed expedient, therefore, to determine the normal expectancy of nucleated erythrocytes in viable newly-born infants when the mother and infant were compatible, *i.e.*, when both were either Rh-negative or both Rh-positive.

Materials and Methods. Human anti-Rh sera were obtained through the kindness of Dr. Phillip Levine and the technic recommended by him was followed. Smears and Rh determinations on the mothers were made routinely within 24 hours after delivery until 116 had been obtained. The smear was stained with Wright's stain. The Jefferson Hospital material was used because this hospital contains only private patients who were assumed to be generally better nourished than charity patients.

The method employed consisted of determining the number of red blood cells in various high power fields until 10,000 were obtained.† Since the high power field is a circle and the area of a circle is $3.1416\ (d/2)^2$ it was only necessary to determine the diameter in each field. The number of red blood cells in the high power field was determined by considering the number in a diameter of the field as the number of red blood cells which a dividing hair touched. The number in the field was considered to be half this diameter squared, multiplied by 3.1416. The diameters of the fields in the 116 smears studied ranged from 12-26 on the various slides. It was found that on a given slide the various diameters had an almost insignificant variation. Consequently in only every fifth field was the diameter determined. Calculation was facilitated by having at hand a chart showing the theoretical number of red blood cells in the field corresponding to each diameter from 12-26. In this manner 10,000 red blood cells were estimated on each slide. The use of the method was validated by actual area counts and approximations within the limits of sampling error were obtained.‡

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* With the assistance of the laboratory staff.

[1] Levine, P., and Stetson, R. E., *J. A. M. A.*, 1939, **113**, 126.

[2] Landsteiner, K., and Wiener, A. S., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 223.

[3] Levine, P., and Katzin, E. M., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 343.

[4] Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

[5] Levine, P., Katzin, E. M., and Burnham, L., *J. A. M. A.*, 1941, **116**, 825.

† The enumeration was first done by using a pointer and counting all nucleated and non-nucleated erythrocytes which the tip of the pointer touched in systematic excursions across the slide. This proved too time-consuming and a simpler method was adopted. It was not believed that the calculation of the number of red blood cells per hundred white blood cells was sufficiently informative to be of much value; if used such a method would require, in addition, very accurate total red and white cell counts.

‡ Theoretically in the extreme case of one cell large enough to occupy a full diameter some 3,000 fields would have to be examined provided the cells were in closest approximation in order to obtain 10,000 red blood cells, since the times when one cell would exactly cover the field would be negligible and inconsequential. To test this possibility a number of circular objects of the approximate size

TABLE I.
Number of Nucleated Erythrocytes per 10,000 Red Blood Cells in 116 Infants with Known Rh Values in Mother and Child.

No. of nucleated red cells per 10,000	Mother Rh+ Infant Rh+	Mother Rh— Infant Rh—	Subtotal	Mother Rh+ Infant Rh—	Mother Rh— Infant Rh+	Subtotal	Grand total
0	59 x	8	67	4	11	15	82
1	15	1	16	0	2	2	18
2	2	1	3	2	1	3	6
3	0	0	0	1	2	3	3
4	0	0	0	1	0	1	1
5	1 i	0	1	0	0	0	1
6	0	1	1	0	0	0	1
7	0	0	0	0	0	0	0
8	1 d	0	1	0	0	0	1
9	1 d	0	1	0	0	0	1
10	1 d	0	1	0	0	0	1
11	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0
13	1 d	0	1	0	0	0	1
Total	81	11	92	8	16	24	116

x—2 died. i—ill. d—died.

Results. Of the 116 pairs of mothers and infants there were 81 instances in which both parent and child were Rh-positive, 11 instances in which both were negative, 16 instances in which the mother was Rh negative and the infant Rh positive, and 8 instances in which the infant was Rh-negative and the mother Rh-positive. The distribution of the nucleated erythrocytes for each 10,000 red blood cells is presented for the four categories (Table I).

Among the 92 infants which had the same Rh value as their mother, 6 died within a few days. Among the 86 viable and Rh compatible infants only 33 nucleated erythrocytes were encountered among an estimated 860,000 red blood cells or an average of 0.39 nucleated red cells per 10,000 red cells. More nucleated red cells than 2 per 10,000 should occur less often than in one of each 100 newly-born normal infants. It will be seen that the infants with the 4 highest nucleated erythrocyte counts died within a few days. It is probable that routine nucleated erythrocyte counts on the newly-born would detect many dyscrasias sufficiently early to institute prompt treatment. By the method described counts of nucleated erythrocytes can be made as quickly as a total white cell count.

Discussion. Among the 24 infants which had a different Rh value than their mother, 7 had two or more nucleated cells in the 10,000 counted; whereas among the 92 infants having Rh values identical with their mother, only 9 had two or more nucleated per 10,000 red blood cells. This difference between the Rh compatible and the Rh incompatible was statistically significant ($X^2 = 6.15$, $n = 0.01$, significant). From the published reports one might assume that this increased percentage of nucleated cells was due to the group in which the mother was Rh-negative and the infant Rh-positive. However, although the tendency was in this direction in both, the real preponderance was in the group in which the child was Rh-negative and the mother Rh-positive. Four of the 8 in this group had 2 or more nucleated red cells per 10,000 as compared with 9 of the 92 with Rh compatibility. Some 30 additional observations would be necessary to test the significance of this tendency.

Summary. A method of making nucleated

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of a field were kept in closest approximation and 20 attempts at random sorting made. From 1-3 cells were found to touch the dividing hair in the 20 instances and the actual number of cells touching the field were 3-4, averaging 3.75. The theoretical number averaged 3.37 and the difference, 0.38 ± 0.65, was not significant. It did not seem worthwhile to make the 3,000 additional samplings necessary to bring the total cells counted to 10,000.

erythrocyte counts per 10,000 red blood cells is presented. Routine counts within 24 hours of delivery are recommended on newly-born infants and more than 2 nucleated red blood cells per 10,000 are to be considered abnormal. Regardless of Rh compatibility the mortality among children with 5 or more nucleated cells per 10,000 was 67% in our small series, although among the Rh incompatible infants there were more nucleated red blood cells than among the Rh compatible group. The possibility of ill effects when the mother is Rh-positive and the infant Rh-negative is suggested by the data.

14421

A Microbiological Method for the Determination of Tryptophane.

R. D. GREENE AND A. BLACK. (Introduced by H. B. Van Dyke.)

From the Biological Laboratories of E. R. Squibb and Sons, New Brunswick, N.J.

The widespread use of bacteria for the assay of the B-complex vitamins has drawn attention to their requirements for amino acids and their possible use as a basis of measurement of these substances. Detailed studies have been made of the amino acids required by *Lactobacillus casei*[1] and *Lactobacillus arabinosus* 17-5.[2] Bacterial methods of assay for the amino acids, especially tryptophane, have been under investigation in this laboratory and during the progress of this work there have appeared publications from other laboratories in which procedures have been outlined[3,4] for the use of *L. arabinosus* 17-5 in the assay of cystine, iso-leucine, methionine, leucine, valine, glutamic acid, threonine, and tryptophane. It seems desirable at this time to present the results of studies on the specificity of *l*-tryptophane for *L. arabinosus* 17-5 and methods for the preparation of proteins for assay. The importance of such studies is apparent since Snell[5] has recently found that indole or anthranilic acid may replace tryptophane for *L. arabinosus* 17-5, a fact which has also been observed in this laboratory.

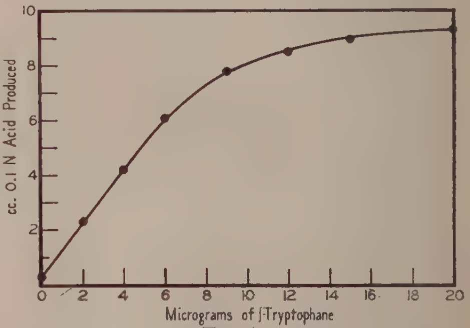

FIG. 1.

Although both *L. casei* and *L. arabinosus* 17-5 require tryptophane, the latter organism was selected for further study because its requirements appeared to be simpler. The Snell and Wright[6] niacin assay provided a technic readily adaptable to the assay of tryptophane. The basal medium differed only in the omission of tryptophane and the inclusion of niacin at a level of two micrograms per assay tube. The preparation and suitable combination of supplements have been described elsewhere.[6,7]

Specificity of Response to Tryptophane. The response of *L. arabinosus* 17-5 to pure *l*-tryptophane (Merck) is shown by a standard curve (Fig. 1) based on about 50 inde-

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[1] Hutchings, B. L., and Peterson, W. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 36.

[2] Shankman, S., *J. Biol. Chem.*, 1943, **150**, 305.

[3] Kuiken, K. A., Norman, W. H., Lyman, C. M., and Hale, Fred, *Science*, 1943, **98**, 266.

[4] Shankman, S., Dunn, Max S., and Rubin, L. B., *J. Biol. Chem.*, 1943, **150**, 477.

[5] Snell, E. E., *Arch. Biochem.*, 1943, **2**, 389.

[6] Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

[7] Greene, R. D., Black, A., and Howland, F. O., *Ind. and Eng. Chem., Anal. Ed.*, 1943, **15**, 77.

TABLE I.
Activity of Pure Substances* for *L. arabinosus* 17-5 in a Tryptophane-free Medium.

Substance	γ per assay tube	% activity (molecular)
l-Tryptophane		100
dl-Tryptophane	5 to 20	48-50
Indole	2 to 8	60-90
Skatole	100	0
Indole-3-acetic acid	100	0
β(Indole-3)-propionic acid	100	0
γ(Indole-3)-n-butyric acid	100	0
Tryptamine hydrochloride	100	0
Anthranilic acid	2 to 8	30-50
Kynurenic acid	100	0

* Tryptophane (*l* and *dl*) was obtained from Merck and Co.; kynurenic acid was a Hofmann LaRoche product obtained through the courtesy of Dr. C. P. Berg, State University of Iowa, Iowa City; the other compounds were obtained from Eastman Kodak Co.

TABLE II.
Apparent Recovery of Tryptophane from Casein* under Various Conditions.

Condition of hydrolysis	Additions to hydrolyzed casein	Apparent % of *l*-tryptophane
5% pancreatin, 24 hr		1.00
,, ,, ,, ,,	0.6% indole	1.47
,, ,, ,, ,,	0.6% indole, followed by ether extraction	0.99
,, ,, ,, ,,	0.6% anthranilic acid	1.33
,, ,, ,, ,,	0.6% anthranilic acid, followed by ether extraction	1.02
5N $Ba(OH)_2$, 4 hr		0.54
,, ,, 7 ,,		0.52
,, ,, 10 ,,		0.51

* Smaco vitamin-free casein containing 8.0% moisture. On a theoretical basis of 15.6% N for pure casein the value for tryptophane would be 14.7% higher.

pendent series. Table I contains data on the activity of a number of substances which are related to tryptophane. A comparison of the activities of the *l*- and *dl*-forms indicates that *d*-tryptophane is not utilized by *L. arabinosus* 17-5. Indole and anthranilic acid were found to be highly effective in replacing *l*-tryptophane whereas various 3-substituted derivatives of indole were entirely inactive. Lugg[8] and Shaw and McFarlane[9] have discussed the possible interfering effect of indole in chemical tests and it thus appears necessary to exclude indole and anthranilic acid during microbiological assays for tryptophane. It has been found possible to separate both compounds from tryptophane by ether extraction at a pH of 4.0 (Table II).

Preparation of Materials for Assay. For the liberation of tryptophane from proteins pancreatic digestion and treatment with sodium or barium hydroxides were investigated. In a study of the digestion of casein with pancreatin, a procedure about the same as the U.S.P. method[10] was employed. A 0.1% solution of casein at pH 8.2 was digested at 37-38°C with pancreatin (Pfanstiehl 1/110) equal to 5% of the weight of the casein. Under these conditions the tryptophane values as followed by microbiological assay reached apparent equilibrium after about 20 hours. After adjustment to a pH of 4.0 with HCl and two extractions with an equal volume of ether the hydrolysate was brought to a pH of 6.8 for assay. Blank tryptophane values for the pancreatin were obtained by its autolysis and assay under the same conditions.

Tryptophane has been considered relatively stable to barium hydroxide[9,11] though subject to racemization. Although preliminary trials of sodium and barium hydroxide digestion of casein gave no striking differences, the latter seemed preferable and was investigated further. It seemed possible that hydrolysis might be conducted under conditions allowing complete racemization of tryptophane without appreciable destruction. A study of the effect of time and concentration of alkali indicated that hydrolysis of casein with 20 volumes of 5 N $Ba(OH)_2$ for about 5 hours in an autoclave at 15 lb pressure was a suitable treatment. After removal of Ba as $BaSO_4$ at a pH of 4.0 and extraction with ether the hydrolysate was prepared for assay by adjustment to a pH of 6.8 and a concentration of casein of 5 mg/cc. Assays of such hydrolysates against *l*-tryptophane standards yielded recovery values about one-half those obtained from pancreatic hydrolysates of casein and on the assumption of complete racemization of tryptophane during alkaline hydrolysis indicated close agreement between the two methods (Table II). It had been found earlier that pure *l*-tryptophane yielded a value close to 50% when assayed after a similar treatment with baryta.

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[8] Lugg, J. W. H., *Biochem. J.*, 1938, **32**, 775.

[9] Shaw, J. L. D., and McFarlane, W. D., *Can. J. Res.*, 1938, **16**B, 361.

[10] U. S. P., 1936, XI, 276.

This microbiological assay procedure for tryptophane appears to compare favorably in sensitivity and reproducibility with similar methods employed for the assay of the B-complex vitamins. A report of its application to a variety of proteins and foods will appear in a later publication.

Summary. Evidence was obtained which indicates that *L. arabinosus* 17-5 may be used for the assay of *l*-tryptophane. Of other related substances tested only anthranilic acid and indole produced a growth response and these are removed by ether extraction.

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[11] Gilman, H., *Organic Chemistry*, II, 1159, 2d Ed.

14422

Studies on the Absorption of Penicillin from the Stomach.*

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In a previous study,[1] penicillin was shown to be absorbed poorly when it was administered by mouth. In view of the fact that penicillin must be injected parenterally at such frequent intervals in the treatment of certain infections caused by gram-positive organisms, it seemed desirable to make further observations on the absorption of this antibacterial agent from the gastro-intestinal tract.

Methods. The sodium salt of penicillin† was dissolved in 0.85% sodium chloride in a concentration of 1,000 Oxford units per cubic centimeter. This standard solution was used in all experiments.

When penicillin was administered by mouth, 20,000 Oxford units were given in 200 cc of tap water. Frequent samples of blood were withdrawn from the anticubital vein and the serum then separated by centrifugalization. All urine passed for a period of 24 hours was stored as individual specimens at 5°C. The concentration of penicillin in these samples was determined by a method described previously.[2] Gastric juice, bile, and succus entericus were obtained by a Miller-Abbott tube; saliva was collected in a sterile container. When these substances were tested for their destructive effect upon penicillin, strains

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* Supported by a grant from the Johnson Research Foundation, New Brunswick, New Jersey.

† Penicillin used in this study was supplied through the courtesy of Dr. George A. Harrop, Squibb Institute for Medical Research, New Brunswick, New Jersey.

[1] Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 425.

[2] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

of both Lancefield group A, beta hemolytic streptococcus and *Staphylococcus aureus* were used as the test organism.

Procedure and Results. It was first necessary to determine the effect of the various gastro-intestinal secretions on the activity of penicillin. The pH of the samples of saliva, gastric juice, bile, and succus entericus was determined and a known amount of penicillin was added to 10 cc of each sample. The tubes were incubated at 37°C for a period of 24 hours, during which time frequent determinations of the concentration of the antibacterial substance were made. Gastric juice was found to inactivate the penicillin rather rapidly, whereas saliva, bile, and succus entericus exhibited no such effect. In general, those specimens of gastric juice with the lowest pH destroyed the penicillin most readily.

It was next necessary to determine what factor in gastric juice destroyed the antibacterial action of penicillin. Pepsin was added to veal infusion broth at a pH of 5 so that the final concentration was 0.2%. When a known amount of penicillin was added to such samples and to tubes containing broth at a pH of 5, the rates of destruction of the penicillin in the two tubes were equal.

The effect of acid on the antibacterial action of penicillin was determined by adding it to samples of veal infusion broth with the pH adjusted to 2, 3, 4, 5, and 7.3 with dilute hydrochloric acid. Penicillin was added to 10 cc samples of each so that the final concentration in each instance was 0.625 Oxford units per cubic centimeter. After the penicillin had been added, half of the sample was refrigerated at 5°C and the other half incubated at 37°C. For a period of 24 hours the concentration of penicillin in these samples was followed closely. A portion of one of these experiments is shown in Fig. 1 and it is at once apparent that at a pH of 4, or lower, there is a rapid loss of penicillin activity at 37°C whereas at icebox temperature the destruction is somewhat delayed. In all experiments a pH of 7.3 was associated with only a slight inactivating effect during the 24 hours of incubation.

As a result of the above observations, it

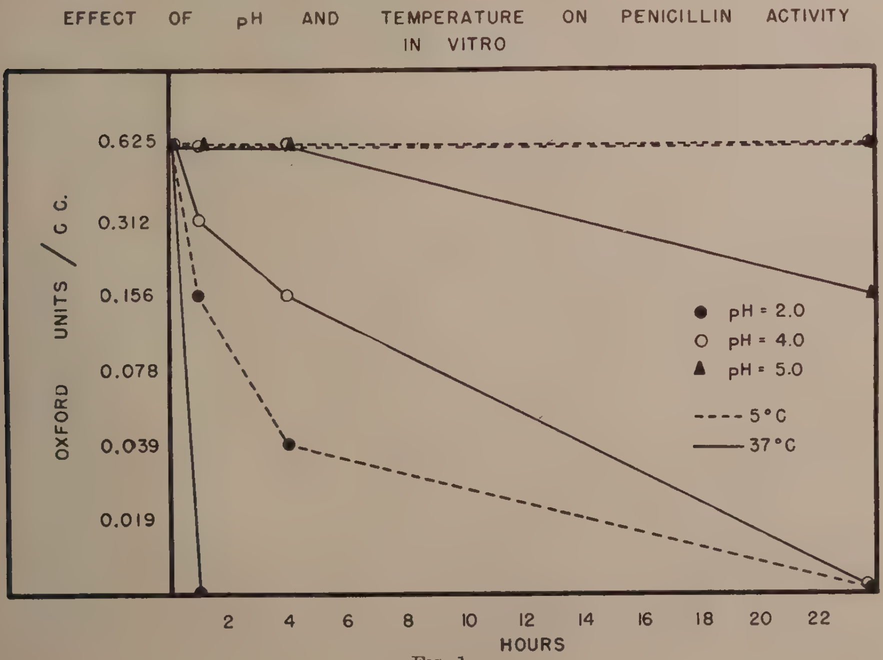

Fig. 1.

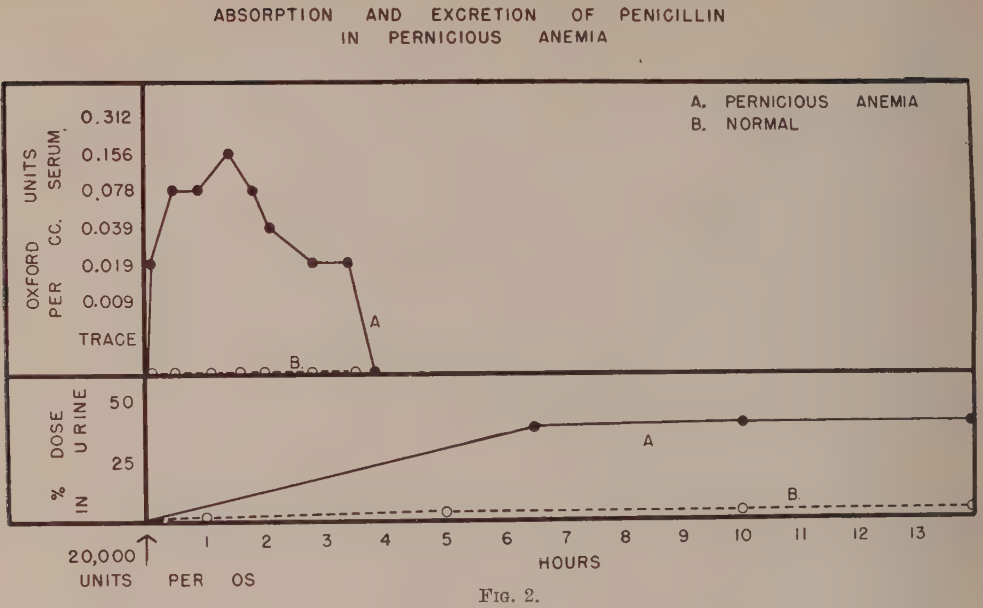

Fig. 2.

seemed apparent that destruction by the acid of the gastric juice was responsible for the apparent poor absorption of penicillin when administered by mouth. If this were true, then, penicillin should be absorbed readily when administered to subjects with achlorhydria. Two patients with the clinical and laboratory findings of classical pernicious anemia were given 20,000 Oxford units of penicillin by mouth. Following the ingestion of this amount in subject A (Fig. 2), the concentration of penicillin in the serum rose rather rapidly to a peak of 0.156 Oxford unit per cubic centimeter. It is of interest that within 10 minutes the serum contained 0.019 Oxford unit per cubic centimeter. At 225 minutes the blood stream showed no antibacterial activity as determined by the test. The first sample of urine, passed 390 minutes after the antibiotic substance was given, was found to contain 7,880 Oxford units. Further specimens yielded only an additional 44 Oxford units.

The results obtained in subject A are in distinct contrast to the observations made in a normal subject (subject B, Fig. 2). In this instance no penicillin was detected in the circulating blood and only 3% of the administered dose was excreted in the urine.

In a second subject with pernicious anemia the total amount excreted in the urine was 4,535 Oxford units after an oral dose of 20,000 units. No blood was obtained for determination of the serum penicillin concentration.

Discussion. Penicillin is absorbed poorly when administered by mouth to normal subjects but if it is administered directly into the duodenum a relatively large amount is absorbed.[1] In one normal subject who received 20,000 Oxford units by mouth both with and without a previous dose of sodium bicarbonate, the concentration of penicillin in the serum and the amount of antibacterial substance excreted in the urine suggested that the acidity of the gastric juice was responsible for the poor absorption observed.[1]

It is well known that acid destroys the activity of penicillin,[3] and these observations have been confirmed by the present study. The inactivating effect of hydrochloric acid was especially marked at a pH of 4 and below, although it may be inhibited somewhat by low temperatures. The specimens of gastric juice studied *in vitro* showed that the lower the pH of the specimen, the greater the inactivating effect. That pepsin was not responsible for the destruction of the penicillin was shown by its failure to cause a decrease in the concentration of the active substance in concentrations of 0.2%.

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[3] Abraham, E. P., Florey, H. W., and Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., and Jennings, M. A., *Lancet*, 1941, **2**, 177.

These studies suggested, then, that penicillin should be absorbed when administered by mouth to subjects with achlorhydria. In 2 patients with pernicious anemia reported herein, the concentration obtained in the serum and the amount excreted in the urine showed that penicillin was absorbed readily. The curve of the plasma concentrations in subject A was not unlike that obtained after intramuscular and intraduodenal administration.[1]

Saliva, bile, and succus entericus did not exhibit any inactivating effect on penicillin, suggesting that oral administration of penicillin might be accomplished effectively by the administration of large amounts of alkali or by the use of enteric coated capsules. The latter method is not likely to prove effective since unless the capsule is dissolved in the upper gut the bacteria in the lower intestines may inactivate the penicillin.[1]

Conclusion. Saliva, bile, and succus entericus obtained from man do not inactivate penicillin. Gastric juice destroys this antibacterial substance rapidly especially at body temperature. Hydrochloric acid, and not pepsin, is apparently responsible for this action of gastric juice.

In 2 subjects with pernicious anemia the absorption of penicillin, when administered by mouth, was greater than that observed in normal subjects.

The authors wish to thank Marjorie Jewell, Thelma Maxon, and Grace Helm for their assistance.

14423

Vascular Fragility and Permeability. II. Influence of Fasting and Bile Salts on Induced Pulmonary Hemorrhage.*

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When normal mice on a regular laboratory diet are subjected to sudden and extreme reduction of atmospheric pressure, they die, and their lungs show extensive hemorrhage. This fact was utilized in testing the ability of certain mixtures derived from lemon or orange peel to decrease capillary fragility.[1]

While comparing the effects of several of these mixtures, some of the mice were left for 24 hours to observe any delayed effect they might exhibit. It was noted, however, that the mice in one lot refused food. When these were killed and post mortem examination revealed absence of hemorrhage, it appeared that the "protection" might be due to fasting. Accordingly, one lot of mice was set aside and deprived of food, but allowed free access to water. At the end of 24 hours they were subjected to the partial vacuum, along with others which had received the regular diet. The fed mice showed the usual hemorrhage of the lungs, but the fasted mice showed much less. By extending the fasting time to 48 hours, the difference between the fasting and fed animals was increased.

The fact that the animals which had been taking food showed hemorrhage, suggested that the effect might be associated with the process of digestion, and prompted an investigation of the role which bile salts might play.

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* Aided by a grant from the California Fruit Growers Exchange and by official W.P.A. Project No. 165-1-07-234.

[1] Majovski, George J., Lesser, A. J., Lawson, Homer C., Carne, Herbert O., and Thienes, C. H., *J. Pharm. and Exp. Therap.*, in press.

The following experiment shows the effect of bile salts on the degree of hemorrhage produced in fasted animals.

Method. The mice selected were adults weighing about 20 g. One lot was fasted for 24 hours, and later lots for 48 hours. After fasting, one-half the lot were given bile salts† in the dosage of 0.1 mg/g body weight, in a 1% solution in distilled water, by stomach tube. The remainder were given an equivalent amount of distilled water. This dosage of bile salts is in accord with the figures of Whipple and Smith[2] for the amount of bile salts circulating in the body. The observation of these authors that there is a lag in the decrease of bile salts after starting a fasting regime, is borne out by the greater effect on hemorrhage of extending the fasting period to 48 hours.[3]

One hour after administering the solution, the mice were placed in the killing chamber. This was a small glass chamber provided with a stop-cock to open and shut a communication with a 5-gallon bottle evacuated by a water pump. A mercury manometer measured the pressure in the system. When the stop-cock was opened, the air pressure in the chamber promptly dropped to about 70 mm of mercury, which is the atmospheric pressure at an elevation of about 54,000 feet above sea level. After 2 minutes they were removed, and the lungs examined. The degree of hemorrhage was estimated, and evaluated in the following manner: lungs showing a normal appearance, of pale pink color, with no bleeding points, no hemorrhagic areas, and no evidence of petechiæ nor extravasation of blood into the pleural cavity, were rated "zero." Lungs showing petechiæ, but no bleeding points nor hemorrhagic areas, were rated "plus-minus." Lungs showing any bleeding points, or hemorrhagic areas involving not more than one-tenth of the total lung area were rated "one plus." Lungs showing hemorrhagic areas involving less than one-half of the total lung area, but of a greater extent than those rated one plus, were rated "two plus." Lungs showing hemorrhagic areas involving one-half or more of the total lung area, but less than virtually the entire lung area, were rated "three plus." Lungs showing hemorrhage involving the entire lung, or all but a minimal area of the lung tissue, were rated "four plus." The average degree of hemorrhage in a series was determined by adding the number of "plusses" (evaluating "plus-minus" as ½), and dividing the total by the number of mouse lungs represented in the series. This figure was multiplied by 25, so that the degree was expressed as per cent, *e.g.*, "four plus" represented total hemorrhage, or 100%.

The abdominal contents were likewise examined. In the fasted animals, the stomach was empty; the gall bladder was distended with

TABLE I.
Pulmonary Hemorrhage in Fasted Mice.

	Bile salts, 0.1 mg/g	No bile salts
24-hr fast	++++	++
	++	+
	++	±
	—	—
% hemorrhage	67	26
48-hr fast	++++	+
	++++	+
	++++	+
	++++	+
	+++	+
	+++	±
	+++	±
	+++	±
	+++	±
	++	0
	++	0
	++	0
	+	0
		0
	—	—
% hemorrhage	73	13
	Bile salts, 0.01 mg/g	
48-hr fast	++++	+++
	++++	++
	+++	±
	+	±
	+	±
	+	±
	±	0
	±	0
	±	0
	±	0
	—	—
% hemorrhage	40	18

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† Difco Laboratories.

[2] Whipple, G. H., and Smith, H. P., *J. Biol. Chem.*, 1928, **80**, 697.

[3] Smith, H. P., Groth, A. H., and Whipple, G. H., *J. Biol. Chem.*, 1928, **80**, 659.

bile, but the intestines showed no color that would indicate the presence of bile.

The results of the experiment are shown in Table I. It is apparent that 0.1 mg of bile salts per gram body weight resulted in the appearance of a much greater degree of hemorrhage in the fasted animals, and that even one-tenth this amount had a definite effect.

Summary. 1. Sudden reduction of atmospheric pressure to 70 mm Hg caused pulmonary hemorrhage and death, in mice. 2. Fasting had the effect of reducing the degree of hemorrhage. 3. Bile salts, given to the fasted animals increased the degree of hemorrhage.

14424

Induction of Decidua-Placental Hemorrhage in Mice by the Endotoxins of Certain Gram-Negative Bacteria.

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Goldman[1] observed that trypan blue intravenously injected localized in parts of the placenta and in certain of the foetal membranes of mice, but not in the embryos. These observations were in general confirmed by Burrows[2] and by Everett.[3] Paralleling these reports many studies have indicated that colloidal acid-azo dyes in the same class as trypan blue, when injected intravenously, localize in traumatized areas, as well as in certain induced or naturally-occurring lesions. Among these studies was the observation that such dyes localize in neoplasms.

Zahl and Waters[4] made a histological study of the localization of various dyes in mouse sarcoma 180, extending similar earlier observations by Ludford,[5] Duran-Reynals,[6] Brunschwig, Schmitz and Clarke.[7] In the course of this study it was observed that certain bacterial products would, when injected into mice bearing implanted sarcoma, induce hemorrhage of the tumor, often leading to its regression.

Initiated by this observation, a series of studies by Zahl, Hutner and co-workers[8] demonstrated that the endotoxins of almost all gram-negative bacteria when injected into tumor-bearing mice would induce tumor hemorrhage, and that this effect was consistent with the view that endotoxins of gram-negative bacteria are primarily vascular poisons.

Stemming from the apparent resemblance between the localization of colloidal dyes in tumors and the localization of such dyes in placental structures, the following study was undertaken to determine whether a parallel also existed in respect to hemorrhage induction in tumors and hemorrhage induction in placental tissues of mice following injection of the endotoxins of gram-negative bacteria.

Experimental. Three representative gram-negative bacterial species were selected as sources of endotoxin.* These were *Shigella*

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[1] Goldman, E. E., *Beitr. z. klin. Chir.*, 1909, **64**, 192.

[2] Burrows, Harold, *Some Factors in the Localization of Disease in the Body*, Bailliere, Tindall and Cox, London, 1932.

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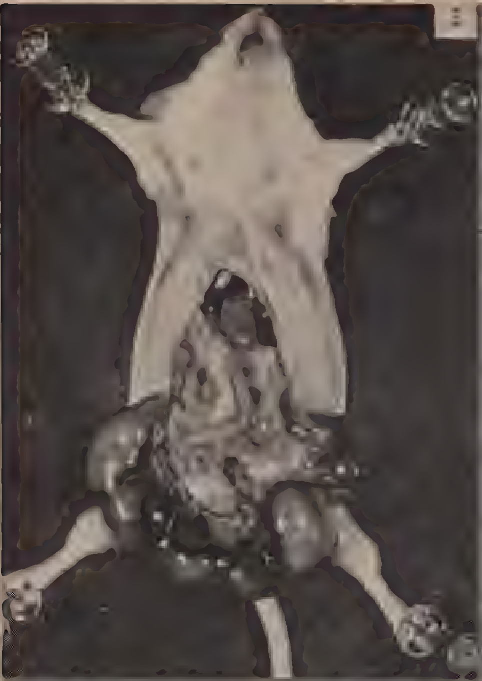
* As many workers now agree that the toxic effects of extracts of gram-negative bacteria are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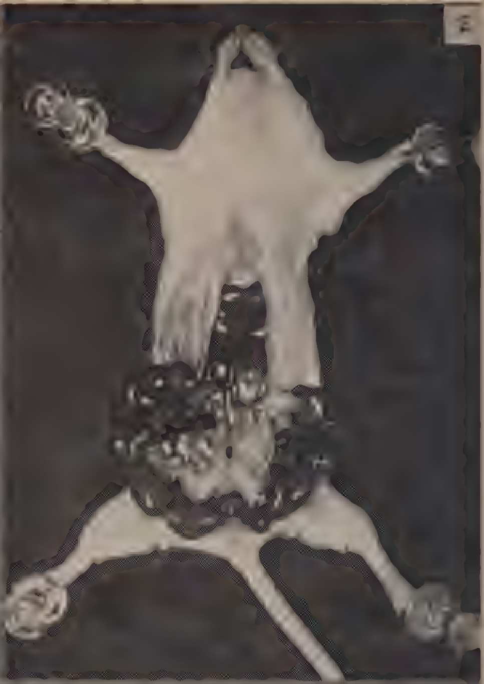
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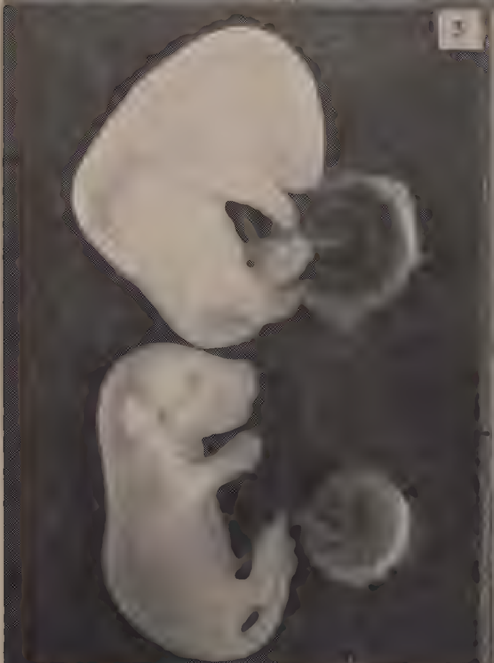
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PLATE 1.

FIG. 1. Pregnant mouse at 18-day stage, sacrificed 5 hours after injection of 1/5 LD50 of *Shigella* extract. Uterus exposed to show hemorrhagic areas. Three embryos from left horn had aborted before time of sacrifice.

FIG. 2. Pregnant mouse at 15-day stage, sacrificed 5 hours after injection of 1/5 LD50 of *Shigella* extract. Uterus exposed to show general hemorrhagic condition.

FIG. 3. Embryos at 18-day stage. Upper figure is of an aborted fetus from mouse pictured in Fig. 1. Note hemorrhage and general purpura in placental discs. Lower figure is of a normal embryo.

FIGS. 4 and 5. Thick unstained sections through 15-day embryos *in situ* in uterus. Fig. 4 shows embryo from female sacrificed four hours after being injected with *Shigella* extract. Fig. 5 shows embryo from normal pregnant female. Note hemorrhagic (A) and non-hemorrhagic (B) placental discs.

paradysenteriæ Flexner, *Salmonella typhimurium,* and *Rhodospirillum rubrum.* The culture and extraction technics, as well as dose-survival curves for each of the three extracts are published in detail elsewhere.[9]

The effect of these endotoxin-containing extracts on mouse placental tissues was studied by injecting an arbitrarily fixed dose of 1/5 LD50. This dose was lethal to none of the mice. About 150 female mice were injected intraperitoneally with the *Shigella* preparation at pregnancy stages corresponding to 12, 15, and 18 days. Length of the gestation was determined by the vaginal plug method.

Within 8 hours following the injection of endotoxin, pregnant mice showed external vaginal bleeding. Occasionally mice failed to show vaginal bleeding, but upon sacrifice and dissection, free blood was to be found in the lumen of the uterus. It appeared that the nearer mice were to term, the more rapid and profuse was the vaginal bleeding.

Mice between 12 days and term showed detachment of the placental disc and evacuation of the embryos from the uterus within 24 hours. Again it appeared that the closer to term such mice were, the more rapid and complete was the abortion. Mice of the 18-day period often aborted within 4 hours after the injection. Mice of the 12- and 15-day stages sometimes exhibited abortion of only a few embryos, the remainder being retained in the uterus and there presumably subjected to slow resorption. In no instance did pregnant mice injected at the stages indicated produce living young. The effect of bacterial endotoxins on stages of pregnancy earlier than 12 days is being studied.

For testing the *Salmonella* and *Rhodospirillum* extracts only 10 animals each were used, these being chosen at about the 15-day stage of pregnancy. These two extracts were tested largely for the purpose of determining whether the effect observed for one gram-negative form would hold for other gram-negative forms not closely related to the first, and thus to ascertain if the hemorrhagic effect on placental tissues would fall into the class of a general effect found to hold for the endotoxins of almost all gram-negative bacteria in respect to hemorrhage induction in implanted mouse tumors. Decidua-placental hemorrhage was induced regularly by the injection of *Salmonella* and *Rhodospirillum* extracts, and was not essentially different from that induced by the *Shigella* extract.

Following the injection of the three endotoxin-containing extracts, the placental disc and the decidual tissues appeared hemorrhagic or purpural in dissected specimens (see Plate I). Such hemorrhage in these tissues apparently resulted in the release of the placental disc from the wall of the uterus, followed by evacuation of the embryos, or by their resorption. At no time was hemorrhage observed in the embryos. Histological observations on such hemorrhagic tissue will be published elsewhere. It has not as yet been ascertained whether hemorrhage appears in such membranes as the yolk sac epithelium, or Reichert's membrane, where dye localization occurs.

Effect of Bacterial Endotoxins on Other Tissues. It is well established that in the guinea pig the adrenal cortex becomes hemorrhagic following injection of the endotoxins of typhoid and other gram-negative bacteria.[10] We could find no evidence of adrenal hemor-

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referable to the endotoxin, the use of this term to describe the active component of our extracts is not considered to involve a liberty in terminology. See reference 9 for detailed discussion of this matter.

[9] Zahl, Paul A., and Hutner, S. H., *Am. J. Hyg.*, in press.

rhage in the mice injected with our bacterial extracts. This is in agreement with Cameron, Delafield, and Wilson[11] who failed to find hemorrhage induction in the mouse adrenal cortex following injection of *Salmonella typhimurium* extract.

It was of particular interest to determine whether the ovary and its component parts exhibited a vascular sensitivity to the endotoxins. In no animals, normal or pregnant, did we observe evidence of abnormal hemorrhage in follicles, corpora lutea, or stroma following injection of bacterial endotoxins; nor did we observe hemorrhage in the normal or the pseudopregnant uterus.

Discussion. It is to be noted that in the last half of pregnancy a dual placenta exists in rats and mice.[3] In addition to the obvious discoidal placenta, there is a villated, vascularized yolk sac placenta. Both of these placental structures constitute barriers or are selectively permeable to variously sized colloidal particles. It is the reaction to the bacterial endotoxins of one or both of these barrier tissues, or of the contiguous uterine tissues, which presumably gives rise to hemorrhage.

Kahler, Shear, and Hartwell[12] have recently reported the molecular weight of the hemorrhagic factor of *B. prodigiosus* to be about 8,000,000. The significance of molecular or of colloidal particle size in this connection cannot be evaluated until more is known about the actual mechanism (1) of colloid passage through the living membrance, (2) of epithelial breakdown by the endotoxin. Furthermore, Boivin[13] in subjecting the purified endotoxin (as O antigen) of *Salmonella typhi* to ultracentrifugation found that both the sedimented and non-sedimented fractions were antigenic and toxic. This would indicate that the activity of such material may be associated with particles of widely varying size. That this applies to the material studied by Kahler, Shear, and Hartwell is indicated by Boivin and Mesrobeanu[14] who have demonstrated a close chemical and physiological similarity between the endotoxins of the species (*S. typhi*) on which they made their centrifugation studies and the species (*B. prodigiosus*) used by Kahler, Shear, and Hartwell. Molecular weight determinations on such materials appear, therefore, in this connection, to be of dubious significance.

Hemorrhage in tumors following injection of the endotoxins of various gram-negative bacteria occurs within 3 to 8 hours.[8] The decidua-placental hemorrhage described in this paper occurs within about that same time interval. This, together with the parallelism in dye localization, suggests strongly that these two hemorrhage phenomena fall into the same class of physiological action.

It has been previously recorded that with relatively unpurified bacterial preparations the minimum dose for hemorrhage induction in mouse sarcoma 180 constitutes about 1/200 minimum lethal dose. In highly purified endotoxin preparations the fraction of the minimum lethal dose necessary to induce tumor hemorrhage may become as low as 1/7000.[8] Whether a comparable fraction of the minimum lethal dose is adequate to induce decidua-placental hemorrhage has not yet been determined. Experiments to ascertain this, as well as to ascertain the effect of bacterial endotoxins on the placental structures of higher mammals, are in progress.

Summary. Endotoxin-containing extracts of *Shigella paradysenteriæ* Flexner, *Salmonella typhimurium*, and *Rhodospirillum rubrum* when injected intraperitoneally into mice pregnant from 12 to 18 days induce decidua-placental hemorrhage which results either in abortion or resorption of the embryos.

It is pointed out that such decidua-placental hemorrhage induced by the endotoxins of gram-negative bacteria may fall into the same class of physiological action as hemorrhage induced in implanted tumors following administration of endotoxins of gram-negative bacteria.

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14425

Hemorrhagic Agent 3,3′-Methylenebis (4-Hydroxycoumarin). V. Its Effect in Prevention of Experimental Thrombosis.*

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The discovery of the hemorrhagic agent 3,3′-methylenebis (4-hydroxycoumarin) (Dicumarol) by Link and his associates[1–5] raised the question whether the reduction it caused in the prothrombin content of the blood would prevent intravascular thrombosis if the intima of the vessel wall had been damaged.

Bingham, Meyer, and Pohle[6] produced such damage by injecting the sclerosing agent Monolate (monoethanolamine oleate) Abbott into a segment of external jugular vein previously clamped off. They permitted circulation for one minute, reclamped, and checked the time necessary for clot formation. An increase in the necessary time was noted in Dicumarol-treated dogs, but no histological studies were done, and they recognized that other variables besides the Monolate were at work.

Dale and Jaques[7] administered 10 mg/kilo of Dicumarol intravenously to dogs. Sixty hours later they crushed the radial and saphenous veins with linen thread, and removed the veins after 2½ hours. They found 60% of the veins free of thrombi in contrast to 100% involvement in the controls.

Murray, Jaques, Perrett, and Best,[8] working with heparin, devised a method for experimental thrombus formation which has recently been used by Richards and Cortell[9] in studies with Dicumarol. The latter investigators used Monolate to produce thrombosis in the radial and saphenous veins in a series of 8 dogs, 4 of which had previously been given fatal doses of Dicumarol, 10 or 25 mg/kilo, daily for 3 to 5 days before injection of the veins with Monolate. Six to 10 days later the veins were removed and examined histologically; thrombi were absent in 10 of 12 veins in Dicumarol-treated dogs, in 3 of 14 veins in the controls.

Using a method similar to that outlined by Murray *et al.*[8] and Richards and Cortell[9] we made a study of the efficacy of Dicumarol in the prevention of venous thrombosis in dogs to which a single dose of the drug had been administered in very safe therapeutic dosage, 5 mg/kilo.

Method. A series of 30 dogs was studied. After the dogs were weighed, the prothrombin time was determined by the Pohle and Stewart[10] modification of Quick's technic on undiluted plasma, and the coagulation time by the 2-tube method of Lee and White at 37°-38°C. On the same day the dogs were matched by weight and divided into 2 groups. Half were kept as controls, and the other 15 were given Dicumarol, 5 mg/kilo, orally, in a gelatine capsule.

Two days later the determinations of prothrombin and coagulation time were repeated, and 0.25 cc of Monolate was injected into a

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* This study has been aided in part by a grant from the Wisconsin Alumni Research Foundation.

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[9] Richards, R. K., and Cortell, R., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 237.

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TABLE I.

Control dogs					Dicumarol-treated dogs				
	Prothrombin time (whole plasma)		Histological study of veins			Prothrombin time (whole plasma)		Histological study of veins	
Dog	Initial	At time of inject. with monolate	Right	Left	Dog	Initial	At time of inject. with monolate	Right	Left
8	5.5	6.5	+	+	7	6	20.5	?	—
10	6	7	+	?	9	6	18.5	—	—
12	6	6.5	+	+	11	6.5	26	—	—
14	6	6	—	—	13	5.5	9.5	?	—
16	5.5	5	?	—	15	5.5	12	—	—
18	5.5	6	+	—	17	6	14.5	—	—
19	5	5	—	+	20	6	36	—	—
21	6	6	+	—	23	6	46.5	—	—
22	5.5	6	—	—	24	6	18.5	?	—
27	5.5	5.5	—	+	25	5	12	—	—
29	5.5	5.5	—	—	26	6	5.5	+	—
30	6	6	—	+	28	5.5	6	?	—
32	6.5	6	+	+	31	6.5	25.5	—	—
33	6	5.5	+	—	34	6.5	22	—	—
36	6	6	+	+	35	6	18	—	+

— No thrombus ? Doubtful + Thrombus present

3-inch segment of each radial vein. During the time of injection and for 3 minutes thereafter, the vein was compressed above and below the site of injection by the fingers, after which compression was released and the sclerosing agent permitted to enter the circulation.

Six to 9 days later, prothrombin and coagulation times were again determined. Then the previously injected segments of veins were removed under morphine and ether anesthesia. Hemostasis was secured by tying off branches to the vein and ligating both ends of the vein before removal. The skin was closed with interrupted sutures. The veins were placed in Helly's solution and histological study was made of all.

The results of the study are listed on Table I. The first group are the controls, the second the Dicumarol-treated animals.

Discussion. Of the 30 veins removed from 15 dogs which had received 5 mg/kilo of Dicumarol orally in a single dose, and which 2 days later had been subjected to the injection of a sclerosing agent, 0.25 cc Monolate (monoethanolamine oleate) Abbott into the radial vein, 2 veins (6.6%) showed definite thrombus formation. Four more (13.3%) were classed as inconclusive with respect to thrombus formation because it was difficult to decide whether the reaction was entirely intimal or whether an organized mural thrombus was present. Thus a maximal total number of 6 veins (20%) showed involvement. In only one (Dog No. 35) of the 2 Dicumarol-treated dogs exhibiting a definite thrombus had the prothrombin time been increased. In one of the 4 exhibiting a questionable thrombus, the drug had not modified the prothrombin level.

In contrast, 15 (50%) of 30 veins in the controls showed thrombi, 2 (6.6%) were listed as inconclusive, the total involved being 17 (56.6%). Bilateral thrombosis occurred in 5 dogs, whereas no bilateral involvement occurred in the Dicumarol-treated group.

No excessive amount of bleeding was encountered at operation, and no post-operative hemorrhage was noted during two weeks of observation in any of the dogs.

Conclusion. A definite reduction in the incidence of thrombus formation was noted in dogs previously treated with Dicumarol in a dosage equivalent to a therapeutic and very safe quantity commonly administered to human beings.

We wish to acknowledge with gratitude the aid of Dr. C. H. Bunting who examined the microscopic sections and confirmed the observations here recorded.

14426

Excretion of Organic Sulfates Following Administration of Adrenalin.

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Several investigators,[1–5] basing their conclusions on experiments carried out *in vitro*, have considered it possible that adrenalin is detoxified in the mammalian body by the oxidative action of enzymes or by autoxidation. Recently however Richter[6] has found conjugation to be the main physiological mechanism by which adrenalin is inactivated in the body. The presence of conjugated adrenalin in the urine of man and the rabbit was shown in his experiments by means of positive tests for adrenalin after hydrolysis with acid. His partially purified specimens gave negative tests for glucuronic acid but positive tests for a sulfate ester.

The following experiment was carried out to furnish quantitative information on the effect of the administration of adrenalin on the excretion of glucuronic acid and organic sulfates.

The urinary sulfates and glucuronates were estimated 24 hours after administration of the

TABLE I.
Excretion of Glucuronic Acid and the Ratio of Inorganic to Total Sulfates in the Urine of Rabbits Following Administration of Adrenalin.

No. of rabbit	Total volume of 1:1000 adrenalin admin., ml	Mode of admin.	Glucuronic acid, mg	Ratio inorganic to total sulfates, %	Comments
1	control	—	10	92	
2	”	—	36	85	
3	”	—	17	90	
4	”	—	59	89	
5	”	—	41	88	
6	1.0	intravenous	36	78	Died 1 hr after final dose
7	1.0	”	40	70	S*
8	1.0	”	18	74	Died 3 ” ” ” ”
9	2.0	”	51	73	S
10	2.0	”	10	73	Died 3 ” ” ” ”
11	2.0	subcutaneous	27	79	S
12	2.5	”	26	75	S
13	2.5	”	37	77	S
14	5.0	”	48	78	S
15	10.0	”	51	64	Died 30 min ” ” ”
16	10.0	oral	48	85	S
17	20.0	”	48	75	S
18	30.0	”	40	64	S
19	40.0	”	29	56	S
20	50.0	”	0	50	S

S = Survived.

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[6] Richter, D., *J. Physiol.*, 1940, **98**, 361.

drug to rabbits, or immediately after death when it occurred within 24 hours. Organic and inorganic sulfates were determined by the turbidimetric method of Treon and Crutchfield,[7] and glucuronic acid by a quantitative procedure described elsewhere.[8] A 1:1000 solution of adrenalin chloride (Parke, Davis and Co.) was employed. It was injected into an ear vein at a rate of 1 ml/30 min. Subcutaneous injections were given in 1.0 ml doses, once every 2 hours, while the compound was given orally (by stomach tube) in 10 ml doses once every 2 hours until the intended dose had been administered. (Precautions to prevent inactivation of adrenalin by reason of the alkaline reaction of the intestine were not taken.)

Experimental results are summarized in Table I. It is evident from the data that adrenalin is responsible for an augmented excretion of sulfate esters in the urine. The intravenous injection of 1 or 2 mg caused a reduction in the percentage of inorganic to total sulfates from a normal average of about 89, to 74%. Doses of 2 to 10 ml injected subcutaneously caused a reduction to 79 and 64%, respectively, while the oral administration of from 10 to 50 ml adrenalin reduced the percentage of inorganic sulfates to 85 and 50, respectively. The excretion of glucuronic acid remained normal after the injection or ingestion of this drug. It is of interest to note that a far greater quantity of organic sulfates was excreted than could be accounted for by conjugation with the amount of adrenalin that had been administered.

Conclusions. In the rabbit the administration of adrenalin is followed by a markedly augmented excretion of organic sulfates. This appears to indicate that this drug is inactivated *in vivo* by conjugation with sulfuric acid. Conjugation with glucuronic acid does not occur. These observations confirm those reported by Richter in 1940.

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14427

On the Toxicity of Atabrine.*

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Atabrine (Quinacrine) has lately attracted much attention as an effective substitute for quinine. In animals large doses of atabrine administered orally cause gastro-intestinal and central irritation; intravenous administration causes lowering of the blood pressure, and direct action of the drug on the excised heart results in "paralysis." In man the gastro-intestinal symptoms are reported to be most conspicuous; irritation of the central nervous system occurs but is encountered more rarely.[1]

A consideration of chemical configuration and the mechanism of drug action suggested that the toxic symptoms encountered after administration of atabrine may be due, in

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* This work was supported by grants from the Joshua Rosett Research Fund and from the Josiah Macy, Jr., and Dazian Foundations.

[1] Findlay, G. M., *Recent Advances in Chemotherapy*, 1939.

TABLE I.
Inhibition of Choline Esterase of the Electric Eel of Human Serum and Red Cells by Atabrine, etc.

Exp. No.	Drug	Concentration molar	γ/ml (free base)	Acetylcholine split per hr, mg	Inhibition %
1				2.81	
				2.82	
	Morphine	$1x10^{-3}$	303.	1.75	38
	”	$1x10^{-4}$	30.	2.46	13
	”	$1x10^{-5}$	3.	2.75	2
2	Quinine	$1x10^{-3}$	324.	1.96	30
	”	$5x10^{-4}$	162.	1.96	30
	”	$2x10^{-4}$	131.	2.22	21
3				5.40	
				5.60	
	Atabrine	$1x10^{-3}$	410.	.51	91
	”	$1x10^{-4}$	41.	.96	82
	”	$1x10^{-5}$	4.	2.43	55
				3.07	
				3.09	
	”	$1x10^{-5}$	4.	1.22	61
	”	$5x10^{-6}$	2.	1.76	43
	”	$2.5x10^{-6}$	1.	2.07	33
4				3.07	
				3.09	
	Eserine	$5x10^{-7}$	.14	.45	85
	”	$2.5x10^{-7}$	.07	.83	83
	”	$1.25x10^{-7}$	.035	.68	68
5		Serum (human) dil. 1:15			
				5.2	
				5.0	
	Atabrine	$1x10^{-3}$	410.	.17	95
	”	$1x10^{-4}$	41.	.43	92
	”	$1x10^{-5}$	4.	1.8	65
6		Red cells (human) dil. 1:25 hemolyzed			
				3.75	
	”	$1x10^{-5}$	4.	1.90	50
		Red cells (human) dil. 1:25 not hemolyzed			
				4.00	
	”	$1x10^{-5}$	4.	2.02	50

part, to the inhibition of choline esterase, the enzyme intimately involved in the transmission of nervous impulses. Support for this hypothesis was obtained in experiments described herewith.

Experimental. The activity of choline esterase was determined with the Barcroft-Warburg manometric method.[2] Preparations of choline esterase from the electric organ of *Electrophorus electricus*, from human plasma and human red cells served as enzyme sources. The original extract of the electric organ was diluted 0.6 to 100, the plasma 1 to 20 and the red cells 1 to 25 with buffer. The red cells hemolyzate was obtained by hemolysis of the washed red cells with 10 times the volume of distilled water. Two ml of the enzyme dilution and 1 ml buffer containing the drug to be tested were introduced in the main vessel. Ten mg of acetylcholine chloride in 0.1 ml buffer was added from the side bulb after thermo-equilibrium had been reached. The effects of atabrine, quinine (bisulfate), morphine (hydrochloride), and eserine (sulfate) on the enzyme were compared. In Table I the mg acetylcholine split during one hour are calculated from the rate of hydrolysis during the first 20 minutes.

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[2] Nachmansohn, D., *Bull. Soc. Chim. Biol.*, 1939, **21**, 761.

Discussion. Atabrine inhibits the choline esterase of the electric organ, of human plasma and red cells to 50% in a molar concentration of 10^{-5} corresponding to 4 γ of free base per ml. Quinine and morphine are on the average 200 times less effective whereas eserine is about 100-200 times more potent calculated on molar concentrations. An equal distribution of a daily dose of 300 mg of atabrine in a man of 70 kg would result in a concentration of 4 γ per g. Since such an equal distribution can not be assumed, the actual effective concentration (disregarding the elimination of the drug) must be much higher in certain tissues. The peripheral toxic symptoms after the administration of atabrine resemble a vagal stimulation, an effect which could be expected from the inhibition of choline esterase.

The particular toxicity of atabrine for the intestinal tract may be an example of selective drug action in the intact organism. The action of a drug depends not only on its affinity for some enzyme, but also on blood supply and on the permeation of the drug into cells and on the interaction of the drug with other cell constituents. For example, physostigmine and prostigmine have the same action in the periphery; but the former acts as a stimulant, the latter as a depressant on the central nervous system.[3] Furthermore, strychnine, although 10-30 times less effective than atabrine in its inhibitory action on choline esterase *in vitro,* acts selectively as a stimulant on the central nervous system.[4]

A study of drug action on isolated enzymes is essential in obtaining a clear picture of the relationship between chemical configuration and drug action. The antimalarials, quinine, atabrine, and plasmochin, are strong inhibitors of the tributyrin splitting lipase.[5,6] The continuous administration of such inhibitors of body esterases may conceivably influence fat metabolism in such a way as to be reflected in the chronic toxicity of these agents.

The chemical configuration which is responsible for the inhibition of choline esterase appears frequently to be related to the presence of an alkyl substituted nitrogen atom. A number of compounds with high affinity for choline esterase, such as methylene blue,[7] oxidation products of adrenaline,[8] and acriflavine[9] contain substituted nitrogen atoms. It is noteworthy that the configuration of one ring nitrogen and 2 nitrogen atoms in a similar side chain occurs in atabrine, plasmochin, and in the antimalarials synthesized by Fourneau.[1] It is also interesting in this connection that methylene blue shows antimalarial activity.

Our finding offers the possibility of an experimental approach to the problem of antimalarials and their toxicity. The specific enzyme inhibiting action found with antimalarials may be used as a lead in synthesizing new and non-toxic drugs. Such a study would show whether the chemical configuration responsible for enzyme inhibiting action is also essential for the antimalarial activity.

Summary. Atabrine is a strong inhibitor of choline esterase. This finding is discussed in relation to toxic and antimalarial activity and chemical configuration of drugs.

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14428

Toxic Levels of Morphine for the Hamster.*

O. Boyd Houchin. (Introduced by Paul W. Smith.)

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In the search for a conveniently small animal in which morphine addiction might be developed preparatory to a study of its effect on tissue metabolism, the hamster was found to be so resistant to the drug that the extremely high lethal dose was thought to be worthy of note. Coincidentally, the toxic symptoms observed differed from those reported in the literature for other species.[1]

The present communication describes the toxic effects of morphine in the hamster. The lethal dose 50 and the lethal dose 100 are presented.

Morphine sulfate was injected subcutaneously, intramuscularly, and intraperitoneally; the route of administration did not alter the response. In all cases where large quantities had to be given at a single time, a combination of the 3 routes was used. Dosage levels ranged from 1 mg to 2000 mg per kilogram body weight. Normal hamsters of both sexes were included. Body weights were from 26 to 130 g with age corresponding from 1 to 8 months (life span of the hamster usually 12 months). Neither sex, age, nor weight of the animal made any apparent difference in the response to morphine. The seasonal or temperature variations during the course of the investigation also caused no difference in response.

In doses up to and including 150 mg per kg of body weight of the hamster, morphine had no noticeable effect. An increase to 300 mg per kg resulted in stimulation of salivary secretion, some incoordination in the extremities, increased activity, and hyperirritability to handling or to any direct contact stimulus. At dosage levels of 500 to 600 mg, the animal also showed some discomfort as indicated by circling movements and by the rubbing of its ventral surface on the floor of the cage. This syndrome was different from the Straub reaction[2] in that there was no arching of the back and the animal was hyperirritable. At this level, convulsions ensued with subsequent recovery. These convulsions, induced by pinching the skin or by blowing the fur, were of the tonic type. Analgesia, as determined by use of heat radiation stimulus, was evident although there was no sedation. As the dose was increased, convulsions became more frequent and severe usually culminating in death. Concurrently, the animal salivated excessively, became damp as if perspiring, and defecated more frequently than normally with semifluid feces. Neither sedation nor narcosis was noted at any time and the respiratory rate was not affected.

By reference to Table I, it will be noted that there were no fatalities in the number of animals used until the dose of 1250 mg per kg body weight was given; this proved to be the L.D. 50 for the hamster. The smallest dose which proved fatal to 100% of the test animals was of the order of 1750 mg per kg; 20 hamsters were included in this group. For only one animal, the guinea pig, has a higher M.L.D. been reported in the literature,[3] although Hatcher and Eggleston[4] have established the M.L.D. for this animal at 400 mg/kg as given by subcutaneous and intramuscular injection and 1000 mg/kg orally.

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* Supported by a grant from the Legislature for research in the University of Oklahoma School of Medicine.

[1] Krueger, Hugo, Eddy, Nathan B., and Sumwalt, Margaret, *The Pharmacology of the Opium Alkaloids*, Part 1, pp. 3-76 and 627-758, United States Government Printing Office, Washington, 1941.

[2] Straub, *Deutsche med. Wchnschr.*, 1911, **37**, 1462, quoted by 1.

[3] Gatti, *Ann. d. Ist. Maragliano p. la cura d. tuberc.*, 1913, **7**, 185, quoted by 1.

[4] Hatcher, R. A., and Eggleston, C., *J. Am. Med. Assn.*, 1914, **63**, 469.

TABLE I.
Toxicity of Morphine Sulfate for the Hamster.

Dose		No. of animals used	No. of survivals	No. of fatalities	Fatality %
Mg/g	Mg/kg				
0.15	150	2	2	0	0
0.30	300	3	3	0	0
0.50	500	6	6	0	0
0.60	600	10	10	0	0
0.90	900	3	3	0	0
1.00	1000	5	5	0	0
1.25*	1250	25	12	13	50
1.50	1500	3	1	2	67
1.60	1600	3	1	2	67
1.70	1700	3	1	2	67
1.75†	1750	20	0	20	100
2.00	2000	3	0	3	100

* The L.D. 50.
† The L.D. 100.

It is quite evident from the above that the hamster is entirely unsuitable for morphine addiction studies. If, by analogy to the dog, the hamster could be addicted to withstand 20 or more times the original lethal dose, it would eventually tolerate around 40 g of morphine per kg body weight per day. On an actual weight basis this would be 4 g for the average adult animal. If given orally, morphine being approximately only one-third as effective by mouth as by injection, would have to replace the entire food intake of the hamster, which is 6 to 10 g.

Since morphine administration produces in this animal a high degree of analgesia without narcosis or respiratory depression, it may prove to be a desirable one in which to study the mechanism of analgesic action. In addition, it was of interest to note that the action of morphine in stimulating salivary secretion, sweating, and defecation was strikingly similar to that of the parasympathomimetic drugs.

14429 P

Hereditary Obesity of Yellow Mice: A Method for the Study of Obesity.*

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In the course of studies on the hereditary obesity of yellow mice first described by Danforth,[1,2] the following observation was made.

A group of 5 obese females was permitted unlimited amounts of water and Purina dog chow pellets throughout the experiment. Their food intake was measured at 2-6-day intervals, and the caloric intake per mouse per day calculated from a value (3.50 cal./g) obtained by chemical analysis. At varying intervals they were also allowed to drink for a few days one of the following at a time: 10% and 20% solutions of dextrose, 50% sucrose, olive oil, and human plasma with a protein concentration of 17.3%. The unrestricted daily caloric intake per mouse of each of these (excepting olive oil) was calculated. Simultaneously, identical steps were taken with a group of 3 normal albino female mice.

Fig. 1 shows the results. Briefly, none of these substances caused a gain in body weight

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* This work was supported by a grant from the Ella Sachs Plotz Foundation.

[1] Danforth, C. H., *J. Heredity*, 1927, **18**, 153.

[2] Rony, H. R., *Obesity and Leanness*, Lea & Febiger, Philadelphia, 1940, 177.

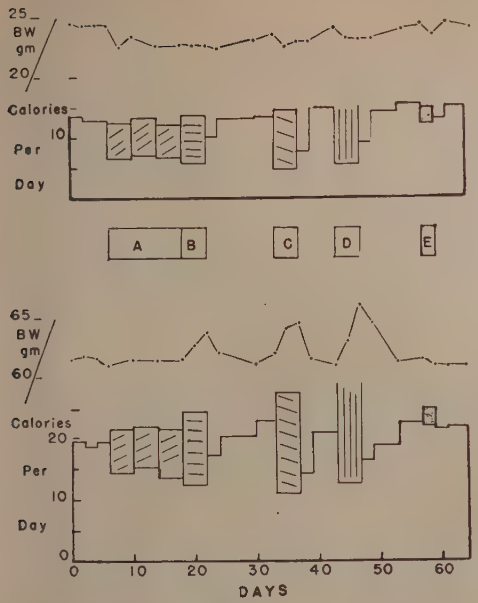

FIG. 1.

Body weight and mean daily caloric intake of 5 obese mice (below) and 3 normal mice (above). Unshaded area, Purina. Shaded area: A, 10% dextrose; B, 20% dextrose; C, 50% sucrose; D, olive oil; E, plasma.

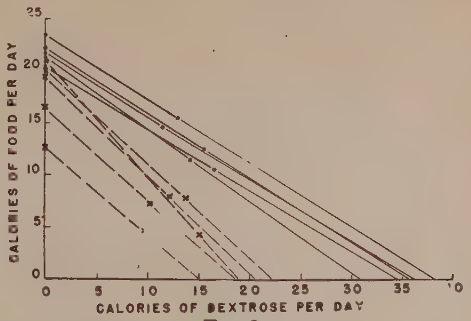

FIG. 2.

Spontaneous substitution of 50% dextrose for Purina. Solid lines, obese female mice; broken lines, normal females. Extrapolations are to emphasize the divergence.

of the normal mice; within the limits of error their caloric intake remained practically constant (see below). But the obese mice gained weight in three 4-day periods: 2.2 g with 20% dextrose, 3.0 g with 50% sucrose, and 4.4 g with olive oil. The caloric intake during 2 of those periods increased by about 12 calories and 24 calories (20% and 50% sugar, respectively), enough to explain most or all of the increased body weight as fat deposition. Moreover, when the sugar or oil was withheld, body weight fell to the original level during a few days in which there was an appropriately reduced caloric intake; surprisingly, under the same conditions, there was also a transient reduction of caloric intake of the normal mice without loss of weight. Dilute (10%) dextrose and plasma protein (17.3%) failed to alter body weight.

Confirmatory observations have been made on 65 mice studied in 20 groups. When "normal" sisters of fat mice are used as controls, some of them gain a gram or two; from this and other evidence (to be published) our present hypothesis is that these animals have "latent" obesity. Excluding such mice, Fig. 2 shows in another manner the results obtained in several groups by the method outlined above, using 50% dextrose, while normal mice substitute 12.2 calories from dextrose for 11.7 calories of their usual diet, the obese substitute 14.1 calories from dextrose for 8.8 calories and so gain in weight.

Among other possible uses of these findings, one seems to have here a method which will permit the study of a fundamental defect of obesity within a short period of time.

14430

Survival of Tubercle Bacilli in Solutions Containing Glycerol or Its Oxidation Products.

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Colorado Springs, Colorado.*

It has been shown by Webb, Boissevain, and Ryder[1] that tubercle bacilli die within a few weeks when their cultures are deprived of oxygen, an observation which was confirmed by Novy and Soule[2] and by Potter.[3]

A suspension of tubercle bacilli in distilled water kept in an anaerobic jar in the incubator becomes non-viable in 3 weeks. The same suspension may remain viable for a year when kept in contact with air. If, however, the tubercle bacilli are suspended in 6% glycerol-water, they die within 2 to 4 weeks in contact with air as well as in the absence of oxygen. Long's medium which is used extensively for the growth of the tubercle bacillus in addition to glycerol contains asparagine, ammonium citrate, phosphate, carbonate, potassium, magnesium, and iron. Addition of these components to the glycerol water does not prevent death of the bacillary suspension unless at least phosphate, potassium, magnesium, and a source of nitrogen have been added; the bacilli then are able to multiply. In the presence of glycerol the bacilli must either multiply or die, while in the absence of glycerol they can be maintained indefinitely in a state of suspended animation.

Experimental. A recently isolated virulent human strain "Voj" was used in the experiments reported here. Most of them have been repeated with 3 other human strains (including H_{37}) giving essentially the same results. A thin veil of a 3-weeks-old culture on Long's medium was washed several times with distilled water and ground to a suspension containing 1 mg bacilli per cc. One-half cc of this suspension was added to test tubes containing 4.5 cc of the solution to be tested, which were then placed in the incubator, and the viability of the tubercle bacilli tested at intervals by adding one drop of the suspension to a flask containing Long's medium. All flasks were carefully cleaned according to the method of Drea,[4] which avoids interference by substances inhibiting growth frequently present on laboratory glassware. The addition of a suspension of tubercle bacilli to Long's medium gives rise to numerous small colonies on the bottom of the flask which subsequently spread to the surface, forming a veil in the usual manner. The use of this technic permits a rough estimate of the number of viable bacteria from the number of colonies appearing on the bottom of the flask. The presence of as little as 10^{-8} mg viable tubercle bacilli can be regularly demonstrated in this manner. When no viable bacilli could be demonstrated, their absence was confirmed by guinea pig inoculation.

A suspension of tubercle bacilli in 6% glycerol water, in distilled water, and in Long's medium minus glycerol were kept in the incubator both in air and anaerobically in a McIntosh jar. Table I shows that all tubercle bacilli suspended in glycerol water had died after 4 weeks in a hydrogen atmosphere while only a few of those in air survived. In water or in Long's medium without glycerol about half the bacilli died in the anaerobic jar while in air there was no appreciable decrease in numbers. After 8 weeks all bacilli in glycerol water had died while a further decrease took place in the number of bacilli in the other mediums kept in the anaerobic jar. The number of bacilli kept under atmospheric pressure had not diminished appreciably.

Bakers c.p. glycerol was used in these

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[1] Webb, G. B., Boissevain, C. H., and Ryder, C. T., *Am. Rev. Tuberc.*, 1924, **9**, 534.

[2] Novy, F. G., and Soule, M. H., *J. Inf. Dis.*, 1925, **36**, 228.

[3] Potter, T. S., *J. Inf. Dis.*, 1939, **64**, 261.

[4] Drea, W. F., *J. Bact.*, 1942, **44**, 149.

TABLE I.
Length of Survival of Tubercle Bacilli in Water and in Glycerol Solutions.

Suspension of washed tubercle bacilli in		No. of colonies in subcultures made after		
		2 wks	4 wks	8 wks
6% glycerol water	(air)	2	1	0
,, ,, ,,	(anaerobic)	2	0	0
Long's medium minus glycerol	(air)	4	4	4
,, ,, ,, ,,	(anaerobic)	3	3	2
Distilled water	(air)	4	4	4
,, ,,	(anaerobic)	3	3	2

4—more than 100 colonies.
3—20-100 colonies.
2—5-20 colonies.
1—less than 5 colonies.

TABLE II.
Comparison of Synthetic and Natural Glycerol.

Suspension of washed tubercle bacilli in		No. of colonies in subcultures made after		
		2 wks	4 wks	8 wks
6% glycerol water, Bakers C.P.	(air)	2	1	0
,, ,, ,, ,, ,,	(anaerobic)	2	1	0
,, ,, ,, (redistilled)	(air)	2	1	0
,, ,, ,, ,,	(anaerobic)	2	1	0
,, ,, ,, (synthetic)	(air)	2	0	0
,, ,, ,, ,,	(anaerobic)	2	0	0
H_2O	(air)	4	4	4
,,	(anaerobic)	4	3	3

4—more than 100 colonies.
3—20-100 colonies.
2—5-20 colonies.
1—less than 5 colonies.

experiments; according to the analysis of the manufacturers it contains 0.03% fatty acids. The soaps of fatty acids and the fatty acids themselves are bacteriostatic for the tubercle bacillus in very low concentrations, and although the fatty acids present in glycerol are probably the short chain fatty acids that have no inhibiting action, the glycerol was further purified by redistillation *in vacuo* and a sample of glycerol was used that had been synthesized from petroleum products and presumably was free from contamination by fatty acids.*

Table II shows that these samples of glycerol had the same effect as Bakers c.p. glycerol.

Since the tubercle bacillus actively metabolizes glycerol the possibility exists that its lack of survival in glycerol water is connected with this metabolism. The effect on the survival of suspensions of tubercle bacilli was studied for the following glycerol metabolites: glycerophosphoric acid, phosphoglyceric acid, glyceric acid, glyceraldehyde, dioxyacetone, lactic acid, and pyruvic acid. A concentration of 0.1% was used in all cases and the solutions were brought to pH = 7.0 with N/1 NaOH. The results are recorded in Table III. Glyceraldehyde and dioxyacetone kill the tubercle bacilli more rapidly than glycerol, while the others are less active, pyruvate being without any lethal effect. The hypothesis that the death of tubercle bacilli in glycerol water is due to the formation of glyceraldehyde is tempting, but its presence could not be demonstrated in the glycerol water suspensions.

Pyruvic acid may be substituted for glycerol in culture media if its concentration

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* We wish to thank the Shell Chemical Co. through whose courtesy we obtained this sample of synthetic glycerol.

TABLE III.
Survival of Tubercle Bacilli in Solutions of Glycerol and Some of Its Derivatives.

Suspension of tubercle bacilli in	No. of colonies in subculture made after 2 wks	4 wks	6 wks	8 wks
6.0% glycerol	2	0	0	0
.1% glyceraldehyde	0	0	0	0
.1% dioxyacetone	0	0	0	0
.1% glycerophosphate	3	3	1	0
.1% phosphoglycerate	2	1	0	0
.1% glycerate	3	3	0	0
.1% lactate	2	1	0	0
.1% pyruvate	4	4	4	4
Distilled water	4	4	4	4

4—more than 100 colonies.
3—20-100 colonies.
2—5-20 colonies.
1—less than 5 colonies.

is maintained below 0.1%. At higher concentrations it inhibits growth. Tubercle bacilli multiply in pyruvate suspensions when Na-phosphate is present. Potassium and magnesium are not required as they are for the glycerol metabolism.

The formation of pyruvic acid in Long's culture medium during the growth of tubercle bacilli was demonstrated through the di-nitro phenylhydrazone. It is apparently an intermediary metabolite in the oxidation of glycerol by the tubercle bacillus.

Glyceraldehyde may be formed from glycerol by removal of 2 hydrogen atoms and pyruvic acid by the removal of 4 hydrogen atoms. Since pyruvate allows the tubercle bacilli to survive, it may well be that this attempted dehydrogenation is the direct cause of death for tubercle bacilli in glycerol water. A similar derangement of the oxidation mechanism may be responsible for the lack of survival of the tubercle bacillus under anaerobic conditions.

Summary. Tubercle bacilli die more or less rapidly in solutions in water of glycerol, glycerophosphate, phosphoglycerate, glycerate, lactate, glyceraldehyde, or dioxyacetone. Similar suspension in distilled water in solutions of inorganic salts and in pyruvate survive for a prolonged period.

14431

Growth Promotion of Bovine Tubercle Bacilli by Pyruvate.

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It has been shown in the preceding paper[1] that human tubercle bacilli die when suspended in a 6% glycerol solution while they remain viable for at least 8 weeks in distilled water or in a one-tenth of one per cent pyruvate solution. If phosphate is added to the pyruvate solution the tubercle bacilli multiply while a similar addition to the glycerol solution has no effect.

Glycerol is believed to be harmful for the growth of the bovine type of tubercle bacilli, and many strains of bovine tubercle bacilli grow very slowly or not at all on Long's medium. Addition of 0.1% pyruvate makes it suitable for the growth of the majority of the dysgonic type of bovine tubercle bacilli.

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[1] Boissevain, C. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 342.

Three strains of bovine tubercle bacilli were isolated on Feldman-Sweany medium from the liver of rabbits that had been injected with an emulsion of tuberculous calf lymph glands.* Transplants from Feldman-Sweany medium to Long's medium gave only very scanty growth and it was impossible to maintain these strains by serial transplants on Long's medium. Addition of 0.1% pyruvate enabled all 3 strains to grow well and even luxuriously on this medium. The pyruvic acid solution was brought to pH = 7.0 with N/1 NaOH, sterilized separately and then added to Long's medium. Addition of crystalline horse serum albumen[2] or of lipoid extract[3] previously described as growth factors for the human tubercle bacillus also enabled these bovine strains to grow on Long's medium. Pyruvate is however without effect on the growth of any of the 12 different human strains tested.

Three laboratory strains of "dysgonic" bovine tubercle bacilli were kindly supplied by Dr. H. J. Corper of the National Jewish Hospital, Denver. One of these strains grew fairly well on Long's medium but better after addition of pyruvate, the second did not grow on Long's medium unless pyruvate was added and the third strain did not grow on Long's medium, with or without pyruvate. Addition of horse serum albumen or lipoid extract had no effect on the growth of this last strain.

Summary. Three newly isolated strains of bovine tubercle bacilli that were unable to grow on Long's medium grew luxuriously on this medium after the addition of 0.1% pyruvate. Of 3 laboratory strains of "dysgonic" bovine tubercle bacilli, 2 showed a similar behavior. Addition of pyruvate had no effect on the growth of 12 human strains of tubercle bacilli.

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* Obtained through the courtesy of Dr. C. L. Davis, U. S. Dept. of Agriculture, Bureau of Animal Industry.

[2] Boissevain, C. H., Proc. Soc. Exp. Biol. and Med., 1942, **44**, 110.

[3] Boissevain, C. H., and Schultz, H. W., *Am. Rev. Tuberc.*, 1938, **38**, 624.

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